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The Role of Nitric Oxide (NO) in the Pathogenesis of Experimental Allergic Encephalomyelitis (EAE)

submitted by Gwen S. Scott
for the degree of PhD
of the University of Bath
1997

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SUMMARY

Experimental allergic encephalomyelitis (EAE) is an autoimmune central nervous system (CNS) disorder which serves as a model for multiple sclerosis (MS). Although CNS inflammation is observed in both EAE and MS, the mechanisms underlying the damage and demyelination are unknown. However, several investigators have proposed a role for nitric oxide (NO), in disease aetiology.

The following investigation aims to further clarify NO involvement in EAE pathogenesis by examining NO production in the CNS throughout the disorder and determining the effect of pharmacological manipulation of NO on neurological disease development. Additionally the CNS generation of reactive oxygen species (ROS) was measured in the model to assess possible effects of altering NO levels on the formation of ROS.

CNS levels of the NO breakdown product, nitrite, were elevated throughout the induction and active phases of EAE. Similarly, protein expression for nitric oxide synthase (NOS), the enzyme which catalyses NO synthesis, was increased during the disease. Furthermore, ROS levels were enhanced in the CNS prior to the onset of symptomatic EAE. Therefore NO and ROS may function as pathogenic effector molecules in disease aetiology. However, CNS nitrite levels were increased during the recovery phase of EAE suggesting that the molecule may also act in a regulatory capacity during the disease process.

Following pharmacological inhibition of NO in EAE, a reduction in CNS nitrite content was not always associated with an abrogation of neurological disease. Thus suggesting that NO is not of primary importance in disease pathogenesis. By comparison, administration of L-arginine, with a resultant increase in CNS nitrite levels, suppressed EAE development implicating a protective effect of NO in disease aetiology.

In conclusion the results of this study indicate that NO may function both in a cytotoxic or cytoprotective capacity during EAE. However, the pharmacological evidence demonstrates that NO acts as a suppressive molecule during the disease process.

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Gwen

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ABBREVIATIONS

7-NI	7-Nitroindazole
AG	Aminoguanidine
BBB	Blood-brain barrier
BH ₄	Tetrahydrobiopterin
BICINE	N,N-bis(2-hydroxyethyl)glycine
bNOS	Brain nitric oxide synthase
BSA	Bovine serum albumin
CFA	Complete Freund's Adjuvant
cNOS	Constitutive nitric oxide synthase
CNS	Central nervous system
CPM	Counts per minute
CREAE	Chronic-relapsing experimental allergic encephalomyelitis
CsA	Cyclosporin A
CTL	Cytotoxic T cell
DAB	Diamino benzidine tetrachloride
Dex	Dexamethasone
DTH	Delayed-type hypersensitivity
EAE	Experimental allergic encephalomyelitis
EAN	Experimental autoimmune neuritis
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FAD	Flavin adenine dinucleotide
FMN	Flavin adenine mononucleotide
hr	Hour/s
i.p.	Intraperitoneally
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin

CHAPTER 1

INTRODUCTION

1. 1 Experimental Allergic Encephalomyelitis

1.1.1 Historical Background

Experimental allergic encephalomyelitis (EAE) is a paralytic autoimmune disease of the central nervous system (CNS), which can be readily induced in certain species of laboratory animals. EAE has served as a model for human demyelinating disorders, such as multiple sclerosis (MS), since the 1930's when it was shown that monkeys given repeated injections of rabbit brain extract developed paralysis accompanied by an acute disseminated encephalomyelitis (Rivers *et al.* 1933). The use of complete Freund's adjuvant (CFA), in the 1940's, allowed EAE to be induced in susceptible animals following a single injection of CNS extract emulsified in adjuvant (Freund *et al.* 1947, Kabat *et al.* 1947). In the 1960's passive transfer of EAE by lymphocytes obtained from sensitised animals was introduced by Paterson (1960) which proved that EAE was primarily a T cell mediated disorder.

1.1.2 Induction and Signs

EAE can be induced in several species and strains of animals using various CNS antigens (Martin & McFarland 1995). However, susceptibility depends on additional contributing factors such as age (Dal Canto *et al.* 1977, Fujinami & Paterson 1977, Ludowyck *et al.* 1993), sex (Jansson *et al.* 1994, Cua *et al.* 1995), and even the commercial source (Swanborg *et al.* 1994) of the animals.

The acute form of EAE, originating from the active sensitisation to CNS antigens has been extensively examined. This can be induced in a number of mammalian species, including guinea pigs, mice, monkeys, rabbits, rats and also some birds (Siller 1960, Paterson 1966, Anderson & Vogel 1961, Lipton & Steigman 1961). Susceptibility to EAE varies considerably between strains. Whereas Lewis rats are highly susceptible to EAE, many others, such as the Brown Norway, are markedly resistant to the disease (Levine & Wenk 1963). Active EAE is usually induced by subcutaneous depot injection of an encephalitogenic emulsion, composed of brain or spinal cord homogenate and CFA,

into the flank, nuchal region or footpad of experimental animals. The encephalitogenic activity of CNS tissues was found to be mainly due to myelin basic protein (MBP), the main constituent of myelin (Kies 1965). Hence, purified MBP is now frequently substituted for CNS tissues in the induction of EAE.

Following inoculation most animals develop an acute monophasic paralytic disease which is terminal in the case of monkeys, rabbits, guinea pigs and mice. However, in the rat the disease is self-limiting and most animals recover (Paterson 1966). Animals sensitised for acute EAE usually develop neurological disease within 10 to 21 days of inoculation. Ataxic gait, paresis or paralysis of both hind limbs, fecal impaction and urinary incontinence are the common manifestations of EAE. However, the type and dose of encephalitogenic antigen, composition of adjuvant, route of inoculation, and species and strain of animal sensitised all influence the course of the EAE.

A chronic-relapsing form of EAE (CREAE) can be induced in certain susceptible strains of guinea pigs and mice (Wisniewski & Keith 1977, Lublin *et al.* 1981). Induction of relapses in the Lewis rat model of EAE has also been achieved by administration of low doses of cyclosporin A (Polman *et al.* 1988) or cyclophosphamide (Minagawa *et al.* 1987).

In addition to active induction of the disease, adoptive transfer of EAE can also be achieved. Lymphoid cells taken from animals previously sensitised to CNS tissue are cultured in the presence of MBP and then injected into histocompatible recipient animals. Following injection the animals develop, within 8 to 10 days, a clinical disease with CNS lesions comparable to those obtained in active EAE (Paterson 1960).

1.1.3 Encephalitogenic Antigens

Following identification of organ specific antigens within the CNS (Witebsky & Steinfeld 1928) the search for encephalitogenic antigens capable of inducing CNS immunopathology began. Potential encephalitogenic antigens are myelin components which are composed of 70 % lipid and 30 % protein (Swanborg 1995). MBP was identified at an early stage as a major encephalitogenic antigen (Kies 1965) and for many

years was thought to be the only autoantigen involved in the pathogenesis of EAE. However, in the late 1970's proteolipid protein (PLP), which constitutes 50 % of myelin protein, was also shown to induce EAE (Sato *et al.* 1987). Although MBP and PLP appear to be the major CNS autoantigens involved in disease other myelin or non-myelin proteins may be of similar encephalitogenic potential to MBP. Indeed other CNS antigens such as myelin oligodendrocyte glycoprotein (MOG) (Amor *et al.* 1994), ganglioside (Cohen *et al.* 1981) and endothelial cell membrane fractions (Tsukada *et al.* 1987) have been shown to elicit EAE. Furthermore, the S 100 protein, an astrocyte-derived calcium binding protein, induces an autoimmune inflammatory response in the CNS (Kojima *et al.* 1994).

1.1.4 Genetics Of EAE

The susceptibility to EAE is genetically determined by well characterised major histocompatibility complex (MHC) coded genes and other non-MHC genes (Pap Hap *et al.* 1988). Indeed the response to a particular encephalitogenic epitope is dependent upon the T cell receptor (TCR) repertoire and MHC class II-restricting elements available to the responder animal. Thus the response of Lewis rats to MBP epitope 68-86 is restricted by RT-1B¹, and the T cells preferentially use V_α2:V_β8 in their TCR (Burns *et al.* 1989, Chluba *et al.* 1989). However, the response of Lewis rats to MBP epitope 87-99 is restricted by RT-1D¹ (Offner *et al.* 1989) and a number of T cell V region gene products are used in the TCR (Sun *et al.* 1992).

In the PLP/J mouse, the response to MBP 1-9 is I-A^U restricted and the T cells responding to this epitope primarily utilise V_β8.2 and V_α2 or V_α4 in their TCR (Zamvil & Steinman 1992). As the SJL/J mouse strain lacks the V_β8 TCR gene family and does not express I-E these animals respond to MBP 89-101 in the context of I-A^S, and 50 % of their T cell clones express the V_β17a TCR (Sakai *et al.* 1988).

The responses of SJL/J mice to PLP 139-151 and 178-191 are I-A^S-restricted (Greer *et al.* 1992), but the responding T cells utilise diverse TCR V genes (Kuchroo *et*

al. 1992). Similarly the response of Lewis rats to PLP peptides is restricted by RT-1B¹ (rat homolog of I-A), but the TCR usage has yet to be determined.

1.1.5 Role of Inflammatory Cells in EAE

1.1.5.1 T Cells

Although the CNS has been considered to be an immunologically privileged site, recent reports have demonstrated that activated lymphocytes can randomly enter the CNS (Hickey 1991). These lymphocytes carry out immunological surveillance and initiate inflammation during infection or autoimmune reactions. However, lymphocytes only remain in the CNS on recognition of their specific antigens (Hickey *et al.* 1991). Furthermore, lymphocyte expression of leukocyte function antigen (LFA)-1 and the up-regulation of its ligand, intercellular adhesion molecule (ICAM)-1, and addressin in brain endothelial cells is required, at least in part, to facilitate lymphocyte entry into the CNS.

Overwhelming evidence demonstrates that EAE is a CD4⁺ T cell-mediated disorder. However, the encephalitogenicity of MBP specific T cell clones is dependent upon the cytokine profile and the ability to express adhesion molecules. T cells can be subdivided depending on the sets of cytokines produced. The Th1 cells function as mediators of delayed-type hypersensitivity (DTH) reactions and produce interleukin (IL)-2, tumor necrosis factor (TNF)- α/β and interferon (IFN)- γ . On the other hand, Th2 cells mainly facilitate humoral immunity and produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Romagnani 1996). In EAE, Th1 cells or Th1-like cytokines have been shown to promote the disease (Kuroda & Shimamoto, Jacobs *et al.* 1991, Selmaj *et al.* 1991,), whereas Th2 cells or Th2-like cytokines suppress disease development (Olsson 1995, Cuo *et al.* 1995). Moreover, MHC class II restricted cytolytic activity has been demonstrated among encephalitogenic T cell lines (Sun & Weckerle 1986, Fallis & McFarlin 1989).

1.1.5.2 B Cells

In addition to T cells, a role for B cells in the pathogenesis of EAE has been suggested. This is demonstrated by the fact that neonatally anti-IgM treated or B cell deficient animals do not develop EAE (Willenborg & Prowse 1983). Moreover, anti-CNS serum or anti-CNS myelin serum from animals with EAE has been shown to induce demyelination *in vitro* (Seil 1994). Further evidence of a role for B cells in EAE aetiology is provided by the observation that EAE progression was prevented by inhibiting B cell activity (Gausas *et al.* 1982).

1.1.5.3 Macrophages

Although CD4⁺ T cells are of primary importance in the induction of EAE, recent reports suggest a pivotal role for macrophages and microglia in the effector pathway of EAE. Indeed *in vivo* depletion of macrophages has been shown to prevent EAE (Brosnan *et al.* 1981, Huitinga *et al.* 1990,1995). Moreover, IL-13, a potent modulator of macrophage function, can suppress the development of EAE without inhibiting either T or B cell immunoreactivity (Cash *et al.* 1994)

Macrophages may mediate damage in EAE through direct effects on myelin (Lampert 1965, Epstein *et al.* 1983). Furthermore activated macrophages may indirectly exert CNS damage through the secretion of numerous factors such as cytokines, proteases, reactive oxygen species (ROS) and nitric oxide metabolites (Nathan 1987), all of which are known to be myelinotoxic and exert cytotoxic effects on oligodendrocytes (Cammer *et al.* 1978, Konat & Wiggins 1985, Selmaj & Raine 1988).

1.1.6 Generation of the Inflammatory Lesion in EAE

The formation of inflammatory lesions during EAE, although not fully characterised, is known to involve several distinct processes. Initially, activated T cells cross the blood-brain barrier (BBB), irrespective of their antigen specificity. This is followed by antigen-specific interaction of invading CD4⁺ T cells with MHC class II positive antigen presenting cells in the CNS, such as microglia and astrocytes. These local

antigen-specific interactions prime the CNS for the induction of an inflammatory response (Weckerle *et al.* 1986). Subsequently, adhesion molecule expression is upregulated on vascular endothelial cells and chemotactic and pro-inflammatory cytokines are released. This facilitates the recruitment of CD4⁺ T cells and activated macrophages into the CNS as well as increasing BBB permeability to serum proteins.

1.1.7 Histology & Pathology of EAE

Neurological EAE is associated with several characteristic histopathological features including breakdown of the BBB, inflammatory cell infiltration, oedema and in chronic forms of the disease, demyelination.

1.1.7.1 BBB Breakdown

The BBB is composed of a continuous layer of endothelial cells sealed together by tight junctions. Astrocytes surround the brain capillaries with their processes and attach to the basement membrane of the endothelial cells. Gaps exist between the astrocyte processes which facilitate the passage of substances across the endothelial barrier. Therefore, astrocytes play an important role in the maintenance of BBB homeostasis (Goldstein 1988). Indeed due to the complex interaction between brain microvessel endothelial cells and the underlying basement membrane-associated cells, such as smooth muscle cells, pericytes and astrocytes, the neuroendothelium is selectively permeable. Moreover, the endothelial cells of the cerebrovasculature possess a number of unique properties which contribute to the functioning of the BBB. Cerebral endothelial cells have intercellular tight junctions, no fenestrae and a reduced number of pinocytotic vesicles (Bradbury 1984). Furthermore the tight junctions which exist between brain endothelial cells have high electrical resistance to limit paracellular diffusion, whereas transcellular flux is restricted by slow-rate fluid phase endocytosis through cerebral endothelial cells (Fabry *et al.* 1994). These specialised characteristics aid the enhanced selectivity of the BBB allowing only the movement of vital nutrients into the CNS (Bradbury 1985, Goldstein & Betz 1986). Consequently exposure of neuronal tissue to vascular

neurotoxins, ion fluxes or other deleterious factors is prevented and homeostasis within the cerebral microenvironment is maintained.

Disruption of the BBB occurs in several pathological conditions of the CNS including EAE and MS. It is thought that the loss in cerebrovascular integrity results from enhanced intercellular leakage caused by increased pinocytosis or tight junction opening (Banks & Kastin 1988). In acute models of EAE it was demonstrated that tight junctions were disrupted, whereas the number of pinocytotic vesicles in brain endothelial cells appeared normal. However, in CREAE models BBB breakdown was shown to be mediated by a metabolic change in the cerebral endothelial cells associated with increased vesicular transport (Moor *et al.* 1994). Furthermore the tight junctions were unaffected during CREAE (Hawkins *et al.* 1992).

Enhanced cerebrovascular disruption is an early event in EAE which is observed to precede the occurrence of cellular infiltration and neurological deficits (Liebowitz 1969, Juhler *et al.* 1986). Indeed it is thought that disruption of the BBB initiates a cascade of events such as oedema formation and inflammatory cell infiltration, which in more chronic disease forms ultimately culminates in demyelination.

1.1.7.2 Inflammatory Cell Infiltration

EAE is characterised histologically by the presence of many perivenular foci of mononuclear cell infiltrates in the spinal cord and brainstem region (Paterson *et al.* 1970, Oldendorf & Towner 1974). Low grade infiltration of T cells into the CNS is observed following EAE inoculation and before the onset of disease signs (Traugott *et al.* 1982). Primary invasion of the CNS is followed by widespread infiltration of large numbers of inflammatory cells prior to neurological disease development (Paterson 1976, Raine 1984). Perivascular infiltrates consist mainly of lymphocytes (Traugott *et al.* 1982), with plasma cells (Lampert 1982) and macrophages (Kristenson *et al.* 1976, Juhler *et al.* 1985) also present.

1.1.7.3 Oedema

Extensive vasogenic oedema develops in the CNS immediately prior to the onset of EAE disease signs (Liebowitz & Kennedy 1972, Oldendorf & Towner 1974). Oedema occurs during EAE as a consequence of BBB dysfunction and vascular permeability alterations (Paterson *et al.* 1987, Claudio *et al.* 1990). Moreover, in EAE paralytic disease signs have been shown to correlate with the extent of oedema in the CNS (Paterson 1976, Raine 1983). Previous studies have suggested that CNS damage during EAE may result from oedema-associated ischemic injury following breakdown of the BBB (Wajda 1972). Evidence exists indicating that an accumulation of fluid within the CNS compartment may cause increased pressure on nerve fibres, resulting in nerve dysfunction and the characteristic neurological signs of EAE (Paterson 1982, Simmons *et al.* 1982).

1.1.7.4 Demyelination

Another histopathological feature of EAE is demyelination. Myelin loss is minimal in acute models of EAE, but animals with CREAE display extensive demyelination (Swanborg 1995). Although the precise mechanisms through which demyelination occurs have yet to be established, several possible routes have been proposed. While some investigators have suggested that myelin destruction may be mediated by cytokines released during CNS inflammation (Brosnan *et al.* 1988), other workers have proposed that demyelination results from a non-specific action of plasma proteins, such as proteolytic enzymes or complement, which enter the CNS following BBB breakdown (Scolding *et al.* 1989). Furthermore, CD8⁺ T cell-mediated killing of oligodendrocytes (Koh *et al.* 1992) and autoantibody-mediated demyelination (Linnington *et al.* 1988) have also been implicated as effector mechanisms in myelin disruption. Similarly, demyelination has been attributed to macrophage effects in the CNS. Indeed microscopy studies have identified two mechanisms through which macrophages may damage myelin. These are: de-lamination via extension of cell processes between myelin lamellae, and lysis caused by the release of soluble factors (Lampert & Carpenter 1965, Wisniewski & Keith 1977).

Finally, recent studies have suggested that oligodendrocyte apoptosis may be an important mechanism leading to demyelination (Tsunoda & Fujinami 1996).

1.1.8 Other Features of CNS Lesions During EAE

Animals inoculated for EAE develop lesions in the CNS which consist of focal areas of perivascular inflammation and demyelination. Interestingly, the white matter of the CNS is involved more severely than the grey and lesions are often found near the ventricular system, in the subcortical white matter and in the brainstem and spinal cord (Paterson 1980). Concomitant with inflammatory cell infiltration during EAE, astrocytes are observed to proliferate and become hypertrophied (Smith *et al.* 1987). In addition, microglia accumulation occurs in the vicinity of the lesions (Matsumoto *et al.* 1986). Furthermore, as chronic EAE progresses gliotic scarring occurs as a consequence of fibrous astrocytes moving into demyelinated areas (Raine 1984, Smith *et al.* 1987).

1.1.9 Regulation of EAE

The loss of neurological disease signs during EAE were initially thought to result as a consequence of remyelination (Pender *et al.* 1989). However, studies have since demonstrated that the transience of symptoms and speed of recovery is not compatible with this mechanism (Simmons *et al.* 1981). Instead investigators have proposed that suppressor T cells may be involved in the regulation of EAE (Swierkosz & Swanborg 1975). Suppressor T cells have been implicated in the spontaneous recovery and subsequent resistance of Lewis rats to the reinduction of EAE (Adda *et al.* 1977, Welch *et al.* 1980, Killen & Swanborg 1982b). These suppressor cells have been characterised as CD4⁺ T cells which produce transforming growth factor (TGF)- β (Karpus & Swanborg 1991) and IL-4 (Karpus *et al.* 1992). Recently Swanborg (1995) has hypothesised that these CD4⁺ suppressor T cells are equivalent to Th2 cells, which diverts the inflammatory Th1 cell response that is thought to initiate the autoimmune pathogenesis associated with EAE. In addition to CD4⁺ suppressor T cells, suppressor CD8⁺ T cells have been isolated from EAE recovered mice (Sun *et al.* 1988). However, these cells have subsequently

been shown only to be involved in the resistance to a second induction of active EAE but not important in the regulation of a first episode of active disease (Jiang *et al.* 1992). Interestingly, it has recently been suggested that a defect in suppressor cell activity may be responsible for the relapses observed in certain animal models of EAE (Weckerle *et al.* 1994, Swanborg 1995).

Alternative explanations for the recovery of animals from EAE also exist. These include the hypothesis that effector cells, such as T cells and macrophages, are eliminated from the CNS by apoptosis (Pender *et al.* 1992). Additionally, MacPhee *et al.* (1989) have proposed that the spontaneous recovery of Lewis rats from EAE is mediated through endogenous corticosteroids with increased circulating corticosterone levels observed in diseased animals prior to recovery. Furthermore, enhanced concentrations of noradrenaline have been detected during the acute stage of EAE implicating a possible involvement of this molecule in the recovery process (Mackenzie *et al.* 1989). Indeed neuroendocrine responses following immune stimulation appear to be important in influencing the course of EAE. The importance of endogenous glucocorticoids in modulating EAE has been further emphasised by depleting circulating steroid levels through adrenalectomy (MacPhee *et al.* 1989), or counteracting their effects by administration of the steroid antagonist, RU486, both of which precipitate a chronic or relapsing form of the disease (Bolton & Flower 1989). Although endogenous corticosterone release in rats with EAE plays an important role in the spontaneous recovery observed in this condition the subsequent inability to reinduce EAE in these animals is not associated with chronically elevated steroid levels (MacPhee *et al.* 1989). Interestingly, more recent studies have demonstrated that CREAE is also regulated by endogenous glucocorticoids with fluctuations in the systemic steroid levels coinciding with the relapsing-remitting phases of the disease (Bolton *et al.* in press).

1.2 Multiple Sclerosis

Multiple sclerosis (MS) is a relapsing-remitting or ultimately progressive CNS disorder which is characterised by areas of demyelination throughout the CNS. Multiple lesions are present, consisting of plaques of demyelination and foci of mononuclear cell infiltration. Lesions develop preferentially within the brainstem, spinal cord, optic nerve and perivascular white matter (Moor *et al.* 1994). During MS neurotransmission is impaired as a result of myelin destruction, and this leads directly to the clinical manifestations of MS, which appear to be dependent upon the area of the CNS in which the lesions develop (Martin *et al.* 1992).

Although the aetiology of MS has not been elucidated, a consistent feature of the disease is the entry of T cells into the CNS (Ffrench-Constant 1994). Therefore, it has been proposed that immunological mechanisms are important in the development of MS (Martin *et al.* 1992).

1.2.1 Comparison of MS and EAE

As the clinical, immunopathological and histopathological features of MS are similar to those seen in EAE (Table 1.1), the experimental model has been widely utilised to gain insight into the mechanisms underlying the human counterpart. Furthermore, various aspects of MS can be examined by using the different models of EAE available. Hence, while acute EAE in the Lewis rat provides an adequate model with which to study the mechanisms underlying the induction and recovery phases of the disease (Weckerle 1993), CREAE enables the immunoregulatory events leading to demyelination and relapse to be defined (Lassman 1983).

Table 1.1 Comparison of EAE and MS

	EAE	MS
Spontaneous disease	TCR ^a transgenic mouse	+
Relapsing and chronic remitting	+	+
CNS demyelination	+	+
CNS inflammation	+	+
Adoptive transfer	+	±
Linkage to MHC	+	+
CD45R-CD4 T cells in CNS	+	+
Neuroantigen-reactive CD4 T cell	+	+
Neuroantigen-sepcific CTL ^b	+	+
Modulation by cytokine	+	+
Restricted TCR gene usage	+	+
Adhesion molecule upregulation	+	+
Role of CD8 T cell	Ts? ^c	?
Anti-myelin antibody	+	+
Role of macrophage	+	?
Apoptosis in CNS	+	?

^a TCR: T cell receptor

^b CTL: cytotoxic T cell

^c Ts: suppressor T cell

(Modified from Tsunoda & Funjinami 1996)

1.3 Pathogenic Mechanisms in EAE

The pathogenic mechanisms involved in the aetiology of EAE are only partially characterised and understood (Figure 1.1). Nevertheless, a clear role for infiltrating cells of the lymphocyte-macrophage lineage together with resident CNS cells has been indicated in disease development. Moreover, invading and resident immunocompetent cells release a variety of inflammatory mediators, including proteases, eicosanoids, cytokines, ROS and nitric oxide (NO), all of which have been implicated in EAE pathogenesis.

1.3.1 Proteases

Proteolytic enzymes are thought to contribute to the inflammatory tissue damage observed in EAE. Not only have proteases been identified in the cerebrospinal fluid (CSF) of mice with EAE (Gijbels *et al.* 1993), they have also been demonstrated to mediate BBB breakdown (Rosenberg *et al.* 1992) and demyelination (Beyer & Whitaker 1985), two of the key processes in the pathology of EAE. A pathogenic role of proteases in EAE is also suggested by the ability of protease inhibitors to suppress EAE (Brosnan *et al.* 1980, Smith & Amaducci 1982, Osani & Nagai 1984). Recently several investigations have proposed a role for matrix metalloproteinases in the pathogenesis of EAE. Pharmacological evidence demonstrates that matrix metalloproteinase inhibitors prevent neurological EAE (Gijbels *et al.* 1994, Hewson *et al.* 1995, Norga *et al.* 1995), thus implicating matrix metalloproteinase formation as a pathogenic effector mechanism during the disease.

1.3.2 Eicosanoids

The arachidonic acid metabolites, prostaglandins and thromboxanes, derived from the cyclooxygenase pathway, and leukotrienes generated via the lipoxygenase pathway are potent inflammatory mediators (Salmon & Higgs 1987, Lewis *et al.* 1990). Although

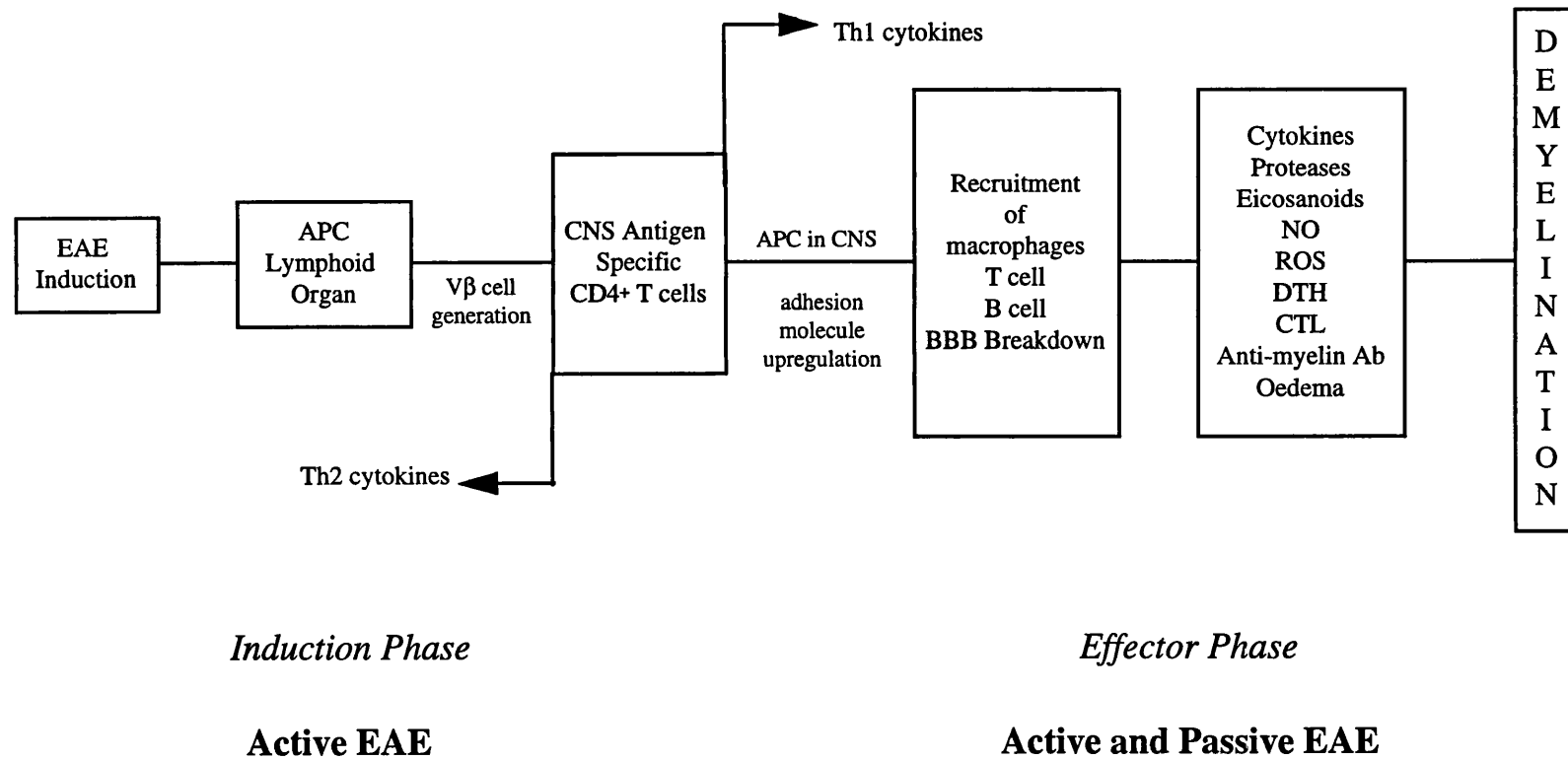


Figure 1.1 Overview of EAE pathogenesis

APC = antigen presenting cell, BBB = blood-brain barrier, NO = nitric oxide, ROS = reactive oxygen species, DTH = delayed type hypersensitivity, CTL = cytotoxic T cell. (Modified from Tsunoda & Fujinami 1996)

the main sources of eicosanoids are macrophages, astrocytes and microglia are also capable of producing arachidonic acid metabolites (Fontana *et al.* 1987, Hartung & Toyka 1987a,b). Arachidonic acid derivatives are known to mediate a variety of inflammatory responses. Indeed, eicosanoids not only function as chemoattractants for neutrophils and macrophages (Turner & Tainer 1983), but also promote adherence of platelets and leukocytes (Rivkind *et al.* 1989). In addition, prostaglandins and leukotrienes enhance vasopermeability and promote oedema (Myers *et al.* 1990a). Furthermore eicosanoids modulate functional activities of T cells and macrophages, thus contributing to immunoregulation (Somers *et al.* 1989). In EAE, increased levels of prostaglandins have been identified in CNS tissues from animals with acute and chronic-relapsing EAE (Bolton *et al.* 1984a,b,1986). Moreover, eicosanoids have been implicated in EAE pathogenesis following the observation that dual cyclooxygenase and lipoxygenase inhibitors attenuated EAE (Weber *et al.* 1991, Simmons *et al.* 1992). Similarly, Reder *et al.* (1994) demonstrated that indomethacin administration suppressed EAE. However, previous work has shown that neurological development of EAE was aggravated by indomethacin treatment (Ovadia & Paterson 1982).

1.3.3. Cytokines

A role for several cytokines including IL-1, IFN- γ and TNF- α in the aetiology of EAE has been postulated.

1.3.3.1 IL-1

Although IL-1 is produced predominantly by macrophages, other cell types such as endothelial cells, microglia and astrocytes can also secrete IL-1 upon stimulation (Arai *et al.* 1990, de Giovine & Duff 1990). IL-1 is a principal participant in inflammatory reactions through induction of other inflammatory metabolites such as prostaglandins, collagenase and phospholipase A₂ (Hartung *et al.* 1989). In addition IL-1 acts on endothelial cells to promote leukocyte adhesion as well as increasing adhesion molecule expression on immunocompetent cells (Frohman *et al.* 1989, Loughlin *et al.* 1992).

Furthermore, IL-1 stimulates other cells to produce various cytokines such as IL-6 and TNF- α (Chung & Benveniste 1990, Bethea *et al.* 1992). Evidence demonstrating a role for IL-1 in the pathogenesis of EAE is provided by the observation that levels of IL-1 were elevated in the CSF of diseased animals (Symmons *et al.* 1987). Similarly, it has been shown that IL-1 immunoreactivity is increased in astrocytes isolated from animals with EAE (Bauer *et al.* 1993, Rubio & Capa 1993). Moreover, immunopharmacological studies have established that administration of IL-1 exacerbated EAE development (Jacobs *et al.* 1991), whereas treatment with an IL-1 receptor antagonist abrogated disease (Jacobs *et al.* 1991, Martin & Near 1995) suggesting a pathogenic role of IL-1 in EAE.

1.3.3.2 IFN- γ

IFN- γ , produced predominantly by T cells also exerts a multitude of inflammatory effects including upregulation of MHC class II and ICAM expression on macrophages and astrocytes (Fierz *et al.* 1985, Sun 1991). Additionally IFN- γ enhances vascular permeability and stimulates the release of ROS from macrophages (Hartung *et al.* 1992). A pathogenic role of IFN- γ in EAE was suggested following the observation that experimental autoimmune neuritis (EAN) was enhanced upon administration of IFN- γ (Hartung *et al.* 1990). However, treatment of EAE with anti-IFN- γ exacerbated the disease, thus suggesting an immunosuppressive effect for the cytokine (Billiau *et al.* 1988). Furthermore, recent studies have reinforced the cytoprotective effect of IFN- γ in EAE (Brod *et al.* 1995). Therefore, the evidence to implicate IFN- γ as a pathogenic mediator of EAE is not substantiated.

1.3.3.3 TNF- α

The major cellular source of TNF- α is from activated macrophages, but other cell types, including T cells, astrocytes and microglia can be stimulated to produce TNF- α (Benveniste 1992). The cytokine enhances endothelial cell permeability (Bredt *et al.* 1989), increases adhesion molecule expression (Pober *et al.* 1986) and facilitates

transendothelial cell migration (Pohlman *et al.* 1986). In addition TNF- α stimulates cytokine production in other cells and modulates the expression of MHC class I and II molecules. Evidence implicating TNF- α as a pathogenic effector molecule in EAE is provided by immunopharmacological studies demonstrating that administration of the cytokine aggravated EAE (Kuroda & Shimamoto 1991) while treatment with anti-TNF- α suppressed disease development (Selmaj *et al.* 1991,5). Moreover TNF- α has been shown to be myelinotoxic (Brosnan *et al.* 1988, Selmaj & Raine 1988) and expression of TNF- α was observed to increase in the CNS during EAE (Renno *et al.* 1995).

1.3.4 ROS

Although ROS are generated mainly by macrophages other cells such as T cell, microglia, astrocytes and neurons are capable of producing ROS (Nathan *et al.* 1983, Colton & Gilbert 1987, Chan *et al.* 1988, Dugan *et al.* 1995). ROS have been proposed as mediators in EAE following the observation that myelin was degraded *in vitro* by ROS (Chia *et al.* 1983, Konat & Offner 1983, Konat & Wiggins 1985). Moreover, ROS may contribute to the CNS damage during EAE through their ability to injure endothelial cells thus disrupting the BBB (Beckman *et al.* 1990, Shukla *et al.* 1992, McQuaid *et al.* 1996). Additionally, pharmacological evidence exists to support a role for ROS in EAE aetiology as interfering with ROS formation has been shown to prevent the disease (Bowern *et al.* 1984, Karlik *et al.* 1991, Lehmann *et al.* 1994, Hansen *et al.* 1995, Ruuls *et al.* 1995). Furthermore other studies have identified increased ROS production during EAE (MacMicking *et al.* 1992, Ruuls *et al.* 1995). Collectively this information provides evidence to substantiate the hypothesis that ROS are involved in EAE pathogenesis.

1.3.5 NO

A role for the highly toxic free radical molecule, NO, in the pathogenesis of EAE has been implicated (see section 1.6).

1.4 Nitric Oxide

Nitric oxide (NO) has been identified as the smallest biologically active molecule produced by mammalian cells. It is a gaseous free radical molecule which is soluble in water and has a tissue half life of 10 to 60 seconds. Due to the presence of an unpaired electron NO reacts readily with oxygen, superoxide and transition metals. Thus NO is capable of mediating a wide range of physiological and pathophysiological actions.

1.4.1 Synthesis of NO

NO is generated following a 5 electron oxidation of one of the terminal guanidino nitrogen atoms of L-arginine (Palmer *et al.* 1988a,b) through a process that incorporates molecular oxygen (Leone *et al.* 1991) and which is catalysed by the enzyme nitric oxide synthase (NOS) (Moncada *et al.* 1991). The process involves two successive monooxygenation reactions, with the initial yielding N^W-hydroxyl-L-arginine as an intermediate (Stuehr *et al.* 1991b) and the final reaction generating NO and L-citrulline (Stuehr & Griffith 1992) (Figure 1.2). Production of NO by NOS requires the presence of the co-substrates L-arginine, nicotinamide adenine dinucleotide phosphate (NADPH) and O₂ as well as being dependent on the existence of a variety of enzyme co-factors. NOS contains 4 prosthetic groups which are involved in NO synthesis: flavin adenine dinucleotide (FAD) (Stuehr *et al.* 1991a), flavin adenine mononucleotide (FMN) (Stuehr *et al.* 1991a), iron protoporphyrin IX (heme) (White & Marletta 1992) and tetrahydrobiopterin (BH₄) (Tayeh & Marletta 1989, Kwon *et al.* 1989). FAD and FMN are involved in the transfer of electrons from NADPH to the catalytic centre (Bredt *et al.* 1991) whereas heme functions as a redox-active co-factor in NOS catalysis (Klatt *et al.* 1992, McMillan *et al.* 1992). Finally BH₄ is necessary for the monooxygenation steps performed by NOS (Stuehr & Griffith 1992).

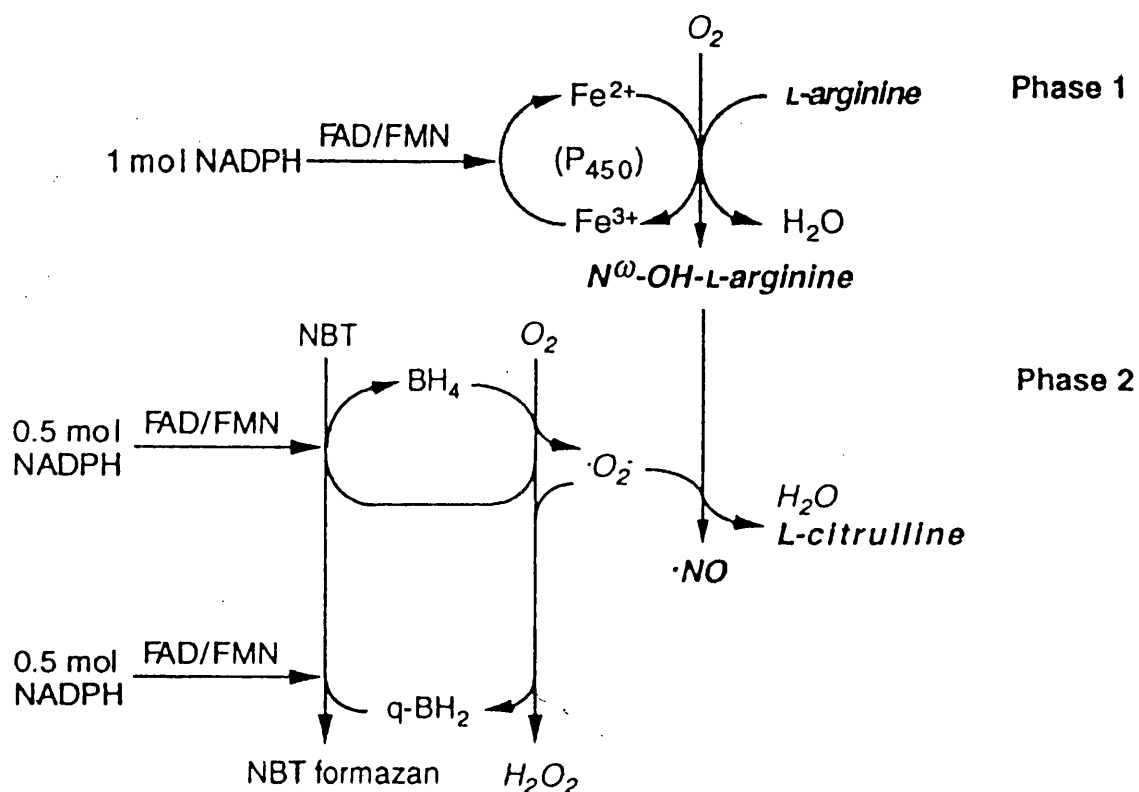


Figure 1.1 Proposed reaction mechanism for NO synthesis.

Molecular oxygen is incorporated into both NO and L-citrulline in at least two phases. The flavin cofactors FAD and FMN, and the cytochrome P₄₅₀ domain of NOS may mediate electron transfer from 1.5 mol NADPH to molecular oxygen in one or both phases. Tetrahydrobiopterin (BH₄) may also be involved in electron transfer and both flavins and BH₄ may then recycle in an NADPH-dependent manner. In Phase 1, L-arginine is hydroxylated to OHArg which requires 1 mol of the electron donor NADPH. In Phase 2, the oxidation of OHArg to L-citrulline and NO requires an additional 0.5 mol NADPH and is dependent on BH₄. (Modified from Schmidt *et al.* 1993).

1.4.2 NOS

NOS activity has been demonstrated in many tissues and cells including endothelium (Furchgott & Vanhoutte 1987), cerebellum (Moncada 1992), macrophages (Nathan 1992) and neutrophils (Wright *et al.* 1989). The NOSs are P-450 like hemoproteins with a reductase domain at the COOH terminus and an oxidative domain at the amino terminus (Wang *et al.* 1993). Three isoforms of NOS have been identified, based on gene sequencing and cloning (Bredt *et al.* 1991, Janssens *et al.* 1992, Lyons *et al.* 1992, Michel & Lamas 1992, Sessa *et al.* 1992, Xie *et al.* 1992). The NOS isoforms vary considerably in their subcellular location, kinetics, regulation and functional role.

1.4.2.1 Constitutive NOS Isoforms

Two of the enzyme types are continuously present and are therefore termed constitutive NOS (cNOS). Activation of cNOS is calcium/calmodulin dependent and the resultant NO production is observed within seconds of administering a cell activation signal (Bredt & Snyder 1989, Förstermann *et al.* 1991). The NO formed by cNOS is usually in picomolar concentrations which are released over a short period of time (Kelm *et al.* 1988, Palmer *et al.* 1988b). This allows the molecule to function as a physiological messenger maintaining homeostasis (Moncada *et al.* 1991).

cNOS have been isolated from endothelial and neuronal cells. Brain NOS (bNOS) was first purified from rat and porcine cerebellum (Bredt & Snyder 1990, Mayer *et al.* 1990). bNOS is invariably a soluble, cytosolic enzyme which migrates with a molecular mass of 150-160 kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Schmidt *et al.* 1991). However, some particulate enzyme activity has been detected in the presence of added FAD (Hiki *et al.* 1992, Matsumoto *et al.* 1993). bNOS have been cloned from rat (Bredt *et al.* 1991) and human (Nakane *et al.* 1993) brain and the deduced amino acid sequences predict proteins of 160 and 161 kDa respectively. Furthermore, the bNOS enzyme appeared to be highly conserved between species with 93 % homology between rat and humans. In addition to the presence of bNOS in the brain, the enzyme is also expressed in other tissues and cells including the spinal cord (Dun *et al.*

1992), peripheral nerves (Sheng *et al.* 1992), epithelial cells (Schmidt *et al.* 1992) and pancreatic islet cells (Schmidt *et al.* 1992).

Endothelial NOS (eNOS) was initially purified from native and cultured bovine aortic endothelial cells and the enzyme has a molecular mass of 135 kDa on denaturing SDS-PAGE (Pollock *et al.* 1991). Unlike bNOS, eNOS is more than 90 % particulate (Förstermann *et al.* 1991) with myristylation at the N-terminal glycine as well as palmitoylation contributing to the membrane association of the enzyme (Pollock *et al.* 1992, Sessa *et al.* 1993, Robinson *et al.* 1995). The cDNA's encoding eNOS have been cloned from bovine (Sessa *et al.* 1992, Lamas *et al.* 1992) and human (Janssens *et al.* 1992, Marsden *et al.* 1992) endothelial cells. The deduced amino acid sequences predict proteins of 133 kDa molecular mass which is in good agreement with that determined by protein purification (Pollock *et al.* 1991). Amino acid sequences for bovine and human eNOS are 94 % identical with both enzymes having a consensus motif for N-terminal myristoylation (Lamas *et al.* 1992, Marsden *et al.* 1992). Although the presence of eNOS is relatively specific for endothelial cells it has also been detected in cells of the human placenta (Myatt *et al.* 1993) and kidney epithelial cells (Tracey *et al.* 1994).

1.4.2.2 Inducible NOS

The third isoform of NOS is not continuously present, instead its expression is induced by certain cytokines such as IL-1, TNF- α and IFN- γ (Stuehr & Marletta 1985, Morris & Billiar 1994) and microbial products (Nussler & Billiar 1993). Therefore the enzyme is referred to as inducible NOS (iNOS). Although iNOS activation is not dependent on calcium following stimulation it takes minutes to hours before NO is generated (Stuehr & Marletta 1987). However, iNOS activity can persist over extended time periods resulting in the production of micromolar concentrations of NO. Due to the formation of high levels of NO, iNOS is thought to be responsible for some of the pathological consequences of inflammation (Moncada & Higgs 1993).

iNOS was first isolated from murine macrophages and the denatured enzyme possessed a mass of 125-135 kDa (Hevel *et al.* 1991, Stuehr *et al.* 1991). Although iNOS

is predominantly soluble some particulate enzyme activity has been identified in induced murine macrophages (Schmidt *et al.* 1992). Several groups have cloned cDNA's for iNOS from a variety of cells (Lyons *et al.* 1992, Xie *et al.* 1992, Adachi *et al.* 1993, Charles *et al.* 1993, Geller *et al.* 1993a, Nunokawa *et al.* 1993, Sherman *et al.* 1993b, Wood *et al.* 1993) and the deduced amino acid sequence predicts proteins of 130-131 kDa. Moreover, different iNOS sequences have been demonstrated in various cells from the same species (Nunokawa *et al.* 1993, Wood *et al.* 1993) but, cDNA sequences were 98 % identical.

1.4.3 Regulation of NOS

1.4.3.1 cNOS

Activity of cNOS is regulated through calcium/calmodulin dependent processes. cNOS is activated by elevations in intracellular free calcium (Ca^{2+}) concentrations. Following receptor stimulation Ca^{2+} influx occurs which results in activation of kinases which subsequently phosphorylate and activate cNOS (Brune & Lapetina 1991, Michel *et al.* 1993, Tsukahara *et al.* 1993). In addition, expressional regulation of both b and eNOS has been identified following brain injury (Herdegen *et al.* 1993, Zhang *et al.* 1994) and sheer stress exposure (Nishida *et al.* 1992) respectively. Although the molecular mechanisms of this regulation are unclear it is thought that sheer stress responsive elements exist in the promotrr region of the eNOS enzyme gene (Marsden *et al.* 1993)

1.4.3.2 iNOS

In contrast to cNOS isoforms, iNOS activity is regulated on a transcriptional level (Kunz *et al.* 1994). The first agents that were found to induce iNOS expression were LPS and cytokines such as IL-1, IFN- γ and TNF- α (Nathan 1992). However the cytokines, alone or in combination, that produce iNOS expression vary between species and cell types (Pfeilschifter *et al.* 1996). In addition agents other than cytokines are capable of inducing iNOS including cAMP-elevating agents (Gilbert & Herschmann 1993) and protein kinase C stimulating compounds (Hortelano *et al.* 1993). There are also a variety

of compounds that prevent iNOS induction such as the inhibitory cytokines IL-4 and IL-10 (Cunha *et al.* 1992, Bogdan *et al.* 1994), monocyte chemotactic protein-1 (MCP-1) (Rojas *et al.* 1993) and TGF- β (Vodovotz *et al.* 1993). Furthermore tyrosine kinase inhibitors (Dong *et al.* 1993) and inhibitors of the activation of the transcription factor NF- κ B (Sherman *et al.* 1993a, Mülsch *et al.* 1993) prevent iNOS induction. This indicates that both tyrosine kinases and NF- κ B are involved in the induction of iNOS.

It is possible to interfere with iNOS expression at several levels from modulation of gene expression to effects on post-transcriptional, translational and post-translational processes. Regulation of iNOS expression at the level of gene transcription is thought to proceed via effects on the promoter region of the iNOS gene (Xie & Nathan 1994). Indeed two regions have been identified as being involved in the induction of iNOS by LPS and IFN- γ (Lowenstein *et al.* 1993b, Xie *et al.* 1993). Moreover, other evidence exists to suggest that transcription factors bind to the iNOS promoter at the NF- κ B site during enzyme induction (Xie & Nathan 1994).

Post-transcriptional and translational control of iNOS has been demonstrated to occur through actions on mRNA stability (Weisz *et al.* 1994). Whereas IFN- γ has been implicated as inducing iNOS by increasing the stability of iNOS mRNA, TGF- β destabilises iNOS mRNA and so decreases translation (Vodovotz *et al.* 1993). Post-translational regulation of iNOS may proceed through effects on substrate and cofactor availability, protein turnover and product inhibition.

As L-arginine is the only substrate for NO synthesis, regulation of its availability could determine cellular rates of NO synthesis. Indeed L-arginine uptake has been shown to increase in macrophages stimulated to produce iNOS (Bogle *et al.* 1992a, Sato *et al.* 1992). Additionally iNOS activity has been shown to be dependent on cofactor availability. More precisely, both BH₄ and NADPH have been demonstrated as regulating iNOS activity (Gross & Levi 1992, Morris & Billiar 1994). iNOS may also be regulated via degradation or inactivation of the functional enzyme (Nathan & Xie 1994). Finally, NO itself has been observed to interfere with the induction of iNOS (Assreuy *et al.* 1993, Griscavage *et al.* 1993). Although the mechanism is not clear it is thought that

NO may bind to and so inactivate the haem moiety of iNOS (Bastian & Hibbs 1994). Moreover, NO may control iNOS gene expression and thus regulate its own synthesis.

1.4.4 Molecular Targets of NO

Analysis of molecular targets of NO has enabled the elucidation of some of the mechanisms of action of NO in homeostasis and host defence. NO's known targets are diverse and include both low molecular weight species as well as macromolecules which can either be activated or inhibited as a consequence of the reaction.

1.4.4.1 Haem Proteins

At low concentrations, NO reacts with Fe²⁺-haemoproteins to elicit many biological actions. For example guanylyl cyclase and cyclooxygenase interaction with NO leads to enzyme activation (Ignarro *et al.* 1982, Salvemini *et al.* 1993). In contrast, NO has been observed to inhibit other haemoproteins such as cytochrome p450 (Wink *et al.* 1993b, Kharitonov *et al.* 1994), thromboxane synthetase, catalase and peroxidase (Gross 1995) which are involved in the synthesis of biological mediators and redox-based cell signalling events. Additionally the interaction of NO with haemoglobin and myoglobin decreases the bioavailability of the active molecule (Furchgott & Vanhoutte 1989).

1.4.4.2 Enzymes

Endogenously produced NO is a potent inhibitor of mitochondrial respiration and energy metabolism (Stuehr & Nathan 1989, Hibbs *et al.* 1990). Moreover the ability of NO to interact with iron sulphur clusters in complex I and II of the mitochondrial respiratory chain and the citric acid cycle enzyme, aconitase, has been linked to the cytostatic/cytotoxic properties of the molecule (Drapier & Hibbs 1988, Stadler *et al.* 1991).

The important glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase is also a target for NO. Inhibition of this enzyme suppresses ATP generation (Molina *et al.* 1992). Indeed the combined inactivation of glycolysis and aerobic mitochondrial

respiration would prevent intracellular ATP synthesis and thus facilitate cell death.

Energy depletion and cell death may also be mediated by NO through activation of poly-ADP ribosyl transferase (Zhang *et al.* 1994a).

1.4.4.3 DNA

NO has been shown to inhibit DNA synthesis. This may be due, in part, to NO-induced inhibition of ribonucleotide reductase (Kwon *et al.* 1991, Lepoivre *et al.* 1991), the rate limiting enzyme of DNA biosynthesis (Elledge *et al.* 1992). Moreover, exogenous NO and NO-generating compounds, when applied at high concentrations, may cause mutagenicity by deamination of DNA (Wink *et al.* 1991). However, this has yet to be demonstrated for endogenous NO.

1.4.4.4 Thiols

Interactions of NO with sulphydryls have been extensively examined (Ignarro 1990). Under aerobic conditions, free thiols react with NO forming S-nitrosothiol compounds (Myers *et al.* 1990b, Clancy & Abramson 1992). These S-nitrosothiol compounds provide the means for storage of bioaccessible NO (Stamler *et al.* 1992). S-nitrosylation has been shown to alter protein activity and it is thought that nitrosation of protein thiols may be fundamental to signal transduction processes (Stamler *et al.* 1992a). Candidate molecules for regulation by thiol-nitrosation include receptor proteins (Lei *et al.* 1992), ion channel proteins (Bolotina *et al.* 1994), G proteins (Lander *et al.* 1993b), protein kinases (Gopalakrishna *et al.* 1993), transcription activating factors (Peunova & Enikolopov 1993) and proteases (Devi *et al.* 1994). Additionally, S-nitrosothiols may limit the generation of toxic free radicals and protect from NO toxicity due to their ability to react with superoxide anions and other ROS (Stamler *et al.* 1992a).

1.4.4.5 Superoxide Anion

Superoxide may be another important target of biologically derived NO. NO reacts readily with superoxide and initially this reaction was thought to represent a detoxification pathway for both molecules (Gryglewski *et al.* 1986). However, the product of this interaction is peroxynitrite which is highly reactive and capable of causing direct and indirect oxidation of biological molecules (Beckman *et al.* 1990, Beckman & Tsai 1994). Therefore, depending on the circumstances, the interaction of NO and superoxide may be cytoprotective or cytotoxic (Darley-Usmar *et al.* 1995).

1.4.5 Physiological Effects of NO

1.4.5.1 Cardiovascular System

NO is produced by the vascular endothelium under basal conditions, and formation of the molecule can be further stimulated by a variety of receptor agonists as well as sheer stress produced by the circulating blood. Basal NO regulates arterial blood flow and pressure by mediating the vasodilator tone (Rees *et al.* 1989). Moreover, vasodilatory agents such as acetylcholine and bradykinin act on endothelial cell surface receptors to trigger NO release and stimulate soluble guanylyl cyclase resulting in smooth muscle relaxation (Rapoport & Murad 1983, Rapoport *et al.* 1983).

The importance of endothelial NO in vascular homeostasis is not limited to its role as a vasodilator. Initially the effects of vascular NO were described as inhibition of smooth muscle contraction (Moncada *et al.* 1991) and prevention of platelet adhesion and aggregation (Mellion *et al.* 1981, Radomski *et al.* 1987). However, additional effects such as inhibition of leukocyte adhesion (Kubes *et al.* 1991), endothelin generation (Boulanger & Lüscher 1990) and smooth muscle cell proliferation (Nakaki *et al.* 1990) are now apparent. Moreover, NO may play a role in controlling heart function. When generated by the coronary vasculature, NO increases coronary blood flow and hence myocardial blood supply (Amezcoua *et al.* 1989). In addition, evidence exists to suggest

that endocardial and myocardial produced NO exerts a negative inotropic effect on heart contractility (Balligand *et al.* 1993, de Belder *et al.* 1993).

1.4.5.2 Respiratory System

Although NO is present in the exhaled air of humans and other mammals (Gustafsson *et al.* 1991) the cellular origin is uncertain. The vasodilator action of NO appears to participate in regulating ventilation-perfusion matching (Persson *et al.* 1990). Moreover, NO may also modulate the contractility of the bronchial tree (Ward *et al.* 1993). Indeed it has been suggested that NO is a neurotransmitter involved in bronchodilation in human airways (Belvisi *et al.* 1992). Similarly NO or an NO-like product is thought to play a role in nonadrenergic noncholinergic (NANC)-mediated tracheal smooth muscle relaxation in a number of different species (Gatson *et al.* 1992, Janson *et al.* 1992).

1.4.5.3 Renal System

In the renal vasculature, the basal tone of the glomerular and medullary microcirculation appears to be regulated by NO (Brezis *et al.* 1991, Zats & de Nucci 1991). Furthermore, the generation of NO in macula densa cells may mediate a vasodilatory component of the tubuloglomerular feedback response and control body fluid homeostasis (Wilcox *et al.* 1992).

1.4.5.4 Gastrointestinal System

NO has been proposed as the mediator of NANC relaxation of nonvascular smooth muscle in the alimentary tract (Bult *et al.* 1990). Therefore, the physiological regulation of peristalsis and intestinal transit may be dependent on NO generation (Calignano *et al.* 1992, Wilklund *et al.* 1993). Additionally, NO may exert cytoprotective effects in the gut against blood-borne toxins and tissue-destructive mediators (Hutcheson *et al.* 1990).

1.4.5.5 Central and Peripheral Nervous System

In the CNS, NO is generated in response to stimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor (Garthwaite *et al.* 1988, Knowles *et al.* 1989). NO formation occurs throughout the CNS (Dawson *et al.* 1992) and NO has been implicated in the regulation of cerebral blood flow (Faraci & Breese 1993) and synaptic plasticity including long-term potentiation (Shuman & Madison 1991), long-term depression (Shibuki & Okada 1991), hyperalgesia (Moore *et al.* 1991) and the development of morphine tolerance (Kolenshikav *et al.* 1992).

In the peripheral nervous system, NO has been proposed as a mediator of sensory transmission (Duarte *et al.* 1990). Moreover, NO has been postulated as a neurotransmitter for peripheral neurons in the oesophagus, stomach, duodenum, ileocolonic junction and penile corpus cavernosum (Garthwaite 1991).

1.4.5.6 Neuroendocrine and Endocrine System

Indirect evidence indicates that NO participates in neuroendocrine function. Indeed, NOS has been detected in the hypothalamus, posterior lobe of the pituitary gland and in the adrenal gland (Palacios *et al.* 1989, Bredt *et al.* 1990, Dawson *et al.* 1992). Additionally, interleukin-2 has been shown to stimulate the release of corticotrophin-releasing factor via a NO-dependent mechanism (Karanth *et al.* 1993). Therefore, NO may regulate hormone release. Moreover, in the presence of D-glucose, pancreatic β cell derived NO stimulates insulin release (Schmidt *et al.* 1992a,b) implicating a role for the molecule in endocrine stimulus-secretion coupling.

1.5 NO in Immunity and Inflammation

1.5.1 Pro-inflammatory Properties of NO

1.5.1.1 Host Defence and Cytotoxicity

NO is widely utilised in the immune system as a cytotoxic or cytostatic agent. Production of NO by activated macrophages contributes to their cytotoxicity against tumour cells, bacteria, viruses, parasitic fungi, protozoans and helminths (Adams *et al.* 1990, Liew *et al.* 1990, Nathan & Hibbs 1991, Karupiah *et al.* 1993). Interestingly this cytotoxic effect of NO provides non-specific immunity against invading organisms and the killing of tumour cells (Drapier & Hibbs 1986, Hibbs *et al.* 1988).

The biochemical basis for NO-induced cytotoxicity or cytostasis may be due to an ability to react with and inhibit iron-containing enzymes of the respiratory chain and DNA synthesis (Hibbs *et al.* 1984, Stadler *et al.* 1991). In addition, NO reacts with and depletes intracellular glutathione, thus increasing susceptibility to oxidant stress (Clancy *et al.* 1994). Furthermore, NO may interact with other free radicals to generate molecules such as peroxynitrite to enhance cytotoxicity (Stamler *et al.* 1992b).

1.5.1.2 Inflammation and Tissue Injury

Increasing evidence indicates that NO may play a part in acute and chronic inflammation or autoimmune tissue injury (Figure 1.3). Indeed NO production is enhanced in inflammatory disorders such as rheumatoid arthritis (Farrel *et al.* 1992), ulcerative colitis (Middleton *et al.* 1993) and diabetes (Corbett *et al.* 1993b). Moreover, treatment with NOS inhibitors prevents the development of certain immune-mediated conditions including arthritis (McCartney-Francis *et al.* 1993), diabetes (Kolb *et al.* 1991), glomerulonephritis (Weinberg *et al.* 1994) and chronic ileitis (Miller *et al.* 1993). NO may contribute to tissue damage through cytostatic or cytotoxic effects on the cells that produce it or neighbouring cells (Moncada 1992).

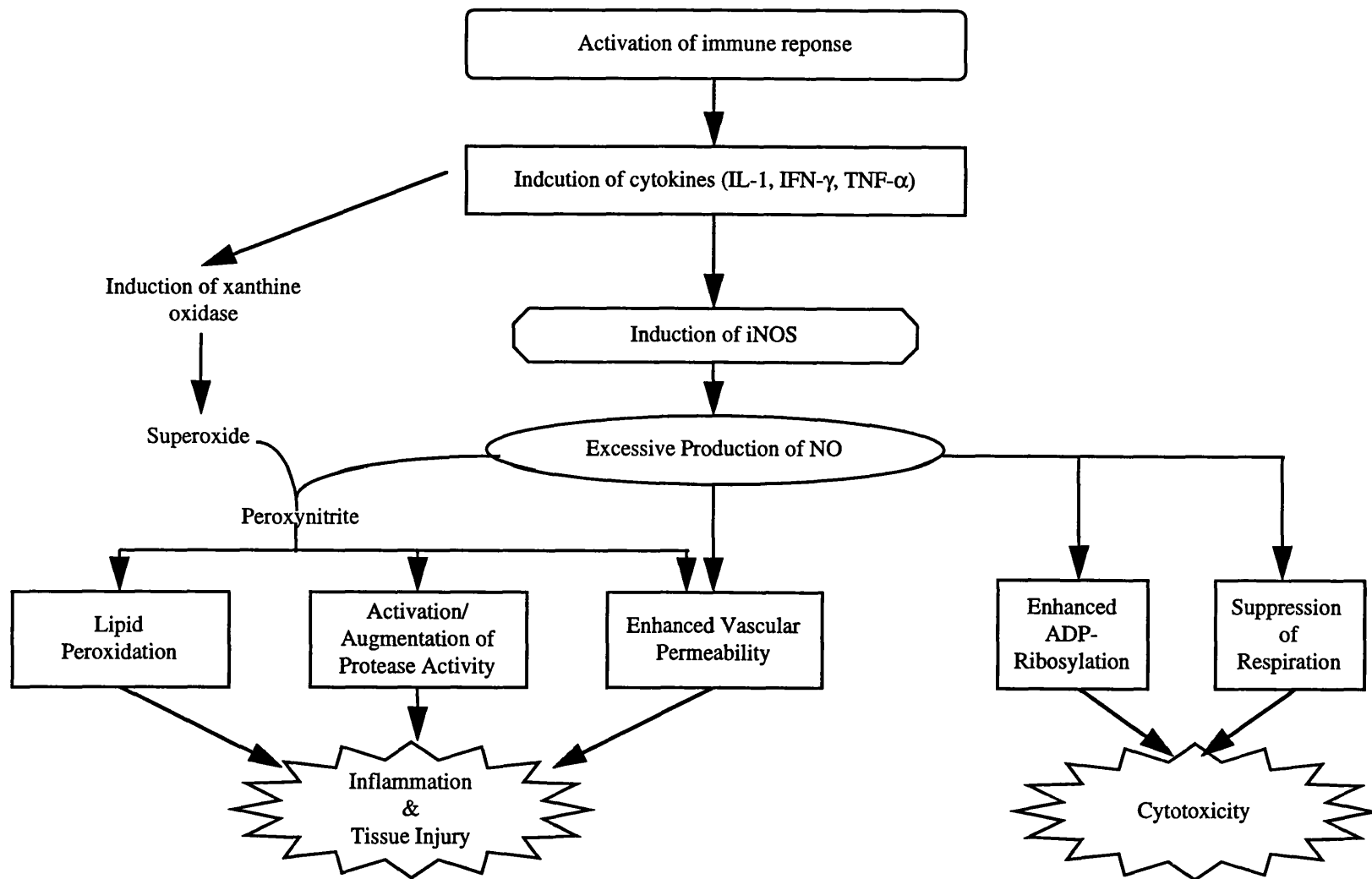


Figure 1.3 Summary of the role of NO in inflammation and immunity (Modified from Maeda *et al.* 1994)

It has been suggested that NO is involved in both the vascular and cellular processes of inflammation. NO modulates the early vascular responses of acute inflammatory reactions as well as mediating the vasodilatation and oedema formation associated with inflammatory processes (Ialenti *et al.* 1992, Laszlo *et al.* 1994). Additionally, NO synthesised by activated immunocompetent cells may regulate the functions of other cells such as neutrophils (Pieper *et al.* 1994), lymphocytes (Albina *et al.* 1991) and peripheral blood mononuclear cells (Lander *et al.* 1993a), which are involved in the inflammatory response. Furthermore, NO may function as a mediator of pro-inflammatory cytokine dependent processes (Clancy & Abramson 1995, Moilanen & Vapaatalo 1995).

1.5.2 Anti-Inflammatory Properties of NO

In addition to pro-inflammatory effects, NO production may serve a protective or anti-inflammatory function. The continuous release of NO is important in maintaining vascular endothelial integrity under physiological and pathological conditions. NO modulates tissue blood flow and regulates interactions between the vascular endothelium and circulating inflammatory cells. The anti-inflammatory effects of NO may be mediated via an inhibition of neutrophil adhesion to the endothelium (Kubes *et al.* 1991), a process required for the development of an acute inflammatory response. Moreover, NO may serve a defensive function by preventing superoxide formation by activated neutrophils (Clancy *et al.* 1992). Similarly NO may also exert an anti-inflammatory effect through the suppression of T-lymphocyte proliferation (Albina *et al.* 1991).

1.6 NO in EAE

In the CNS basal levels of NO are generated by cNOS enzymes present in endothelial cells (Tomimoto *et al.* 1994), neurons (Garthwaite 1991) and astrocytes (Murphy *et al.* 1993). However, endothelial cells, neurons, astrocytes and glial cells are also capable of producing large amounts of NO upon cytokine-mediated induction of iNOS (Boje & Arora 1992, Galea *et al.* 1992, Simmons & Murphy 1992, Minc-Golomb & Schwartz 1994). Following the CNS infiltration of immunocompetent cells during EAE and the initiation of a local inflammatory response, the potential for NO formation must be considerable. Therefore, it is possible that iNOS induction and NO production may be involved in the aetiology of EAE. Indeed Koprowski *et al.* (1993) demonstrated the expression of iNOS mRNA in CNS tissues during EAE. Moreover, MacMicking and co-workers (1992) identified an elevated secretion of reactive nitrogen intermediates in cells isolated from the CNS of EAE-diseased animals. Similarly, increased nitrite levels have been observed in serum samples from EAE-sensitised rats (Ludowyck *et al.* 1993). Furthermore, NO has been localised in spinal cords of mice with EAE (Lin *et al.* 1993). In addition to direct evidence implicating a role for NO in EAE pathogenesis other studies have suggested that both oligodendrocytes and neuronal cytotoxicity are mediated via the production of NO (Demerlé-Pallardy *et al.* 1993, Merrill *et al.* 1993). Therefore further work was undertaken to define the precise role of NO in the pathogenic mechanisms of EAE.

1.7 Aims

The following investigation aims to further define the role of NO in EAE pathogenesis. Using an actively-induced model of EAE in the Lewis rat the importance of NO in disease initiation and development was assessed. In order to accomplish this the following objectives had to be met:

- to devise an efficient method for measuring NO production in CNS tissues to determine differences between samples from normal and EAE-diseased rats.
- pharmacologically manipulate CNS levels of NO to monitor the effect on disease development.
- utilise specific NOS inhibitor to ascertain which isoform/s of NOS may be important in EAE aetiology.
- assess possible interactions occurring between ROS and NO during EAE by measuring CNS levels of superoxide and hydrogen peroxide in the presence or absence of drug administration.

CHAPTER 2

METHODS

2.1 EAE

2.1.1 Animals

Male Lewis rats obtained from the University of Bath Animal House, original breeding colony from Bantin and Kingman (Hull), were used in all experiments. The rats were housed with no more than 6 rats per cage (weight range 200 - 300 g), in temperature controlled rooms (22 ± 2 °C) on a 12 hour light/dark cycle (light period: 6 am - 6 pm) and maintained on Labsure CRM rat diet (containing 0.95 % arginine) and tap water *ad libitum*. Animals were randomly grouped and transferred to the experimental room prior to the beginning of an experiment. A minimum of 6 rats were used per experimental group. All procedures were carried out in accordance with the Animal (Scientific Procedures) Act 1986.

2.1.2. Induction of EAE

EAE was induced using an inoculum composed of 50 mg of guinea-pig spinal cord, 500 µl Freund's incomplete adjuvant and 500 µl sterile, filtered 0.001 M phosphate buffered saline (PBS) pH 7.2, supplemented with 10 mg/ml heat killed *Mycobacterium tuberculosis* H₃₇RA. The inoculum was prepared by chopping the guinea-pig spinal cord in PBS with scissors and briefly homogenising the mixture by repeated trituration with a 1 ml syringe. Following the addition of *M. tuberculosis* and Freund's incomplete adjuvant, the mixture was vigorously shaken and then emulsified by repeated passage between two 20 ml syringes connected by a short length of rubber tubing. The process was continued until the inoculum reached a thick consistency and did not disperse when a drop was floated on a beaker of water. For the induction of EAE, rats weighing 200-250 g received 0.1 ml of the inoculum in each hind foot pad.

In some experiments control animals were injected with an inoculum in which sterile PBS was substituted for guinea-pig spinal cord. Thus the inoculum contained an emulsion of 500 µl Freund's incomplete adjuvant and 1ml sterile PBS plus 10 mg/ml *M. tuberculosis*. These were referred to as complete Freund's adjuvant (CFA) control rats.

2.1.3. Assessment of EAE

Following inoculation animals were weighed daily and examined for neurological signs of EAE from day 7 post-inoculation (PI). The severity of the disease was assessed and graded by assigning a numerical score from 0-4 using the following criteria.

- 0 Animals with no signs of disease.
- 1 Flaccid tail was characterised by a drooping and immobile tail upon raising the rat briefly by the base of the tail.
- 2 Hind limb hypotonia was indicated by an ataxic gait and weak support of hind limbs.
- 3 Rats with only one leg completely paralysed, or both legs moving but neither functional were categorised as having partial hind limb paralysis.
- 4 Animals displaying complete hind limb paralysis.

2.2. Administration of Drugs

All drug administration was carried out by the University of Bath Animal House staff. A summary of drug administration is given in Table 2.1. Control animals received the appropriate vehicle using the applicable dosing regime.

2.2.1 Dexamethasone (Dex)

EAE-inoculated animals were injected subcutaneously (s.c.) with dexamethasone sodium phosphate, diluted in sterile PBS, at a dose of 1 mg/kg body weight. The rats were dosed twice daily for 2 days from the onset of disease related weight loss.

2.2.2 Cyclosporin A (CsA)

CsA was suspended in extra virgin olive oil and administered orally at a dose of 50 mg/kg body weight to rats inoculated for EAE. Dosing was commenced on the day of disease related weight loss for 2 days.

2.2.3. N^G-nitro-L-arginine Methyl Ester (L-NAME)

L-NAME was dissolved in sterile PBS and administered intraperitoneally (i.p.) to EAE-inoculated rats at a dose of 30 mg/kg body weight. Dosing was carried out for 6 days commencing on day 7 P.I.

2.2.4. 7-Nitroindazole (7-NI)

EAE-sensitised animals were dosed i.p. with 7-NI at 10 mg/kg body weight suspended in peanut oil. The rats were dosed once daily from day 7 to 12 P.I inclusive.

2.2.5. Aminoguanidine (AG)

The selective iNOS inhibitor, AG, was prepared in sterile PBS and administered s.c. to EAE-sensitised animals at a dose of 200 mg/kg or 400 mg/kg body weight. Animals were injected once daily from day 1 to 12 P.I.

2.2.6. MK-801

The non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 was suspended in sterile PBS. EAE-inoculated rats were dosed once daily i.p. on days 7-12 P.I. inclusive with 0.3 mg/kg body weight MK-801.

2.2.7. L-arginine

EAE-inoculated rats were orally dosed with 300 mg/kg body weight L-arginine dissolved in sterile PBS. Animals were dosed once daily from day 1 to day 12 P.I. In addition, normal animals received either L-arginine or vehicle according to the same schedule.

Table 2.1 Summary of drug administration protocols

Drug	Dose (mg/kg body weight)	Route of Administration	Vehicle	Treatment Period (Days P.I.)
Dex	1	s.c.	PBS	From weight loss for 2 days
CsA	50	oral	Extra virgin olive oil	From weight loss for 2 days
L-NAME	30	i.p.	PBS	7 - 12
7-NI	10	i.p.	Peanut oil	7 - 12
AG	200, 400	s.c.	PBS	1 - 12
MK-801	0.3	i.p.	PBS	7 - 12
L-Arginine	300	oral	PBS	1 - 12

2.3 Preparation of Samples

All blood and CNS samples were collected the day after the last drug dose.

2.3.1 Sera

Anaesthesia and the collection of blood samples was carried out by the University Animal House technicians. Samples were collected in the morning when plasma corticosterone levels are at their lowest to limit fluctuations in corticosterone caused by circadian variation (D'Agostino *et al.* 1982). The animals were anaesthetised with a mixture of halothane/O₂ and 5 ml of blood was collected by cardiac puncture. The rats were then asphyxiated with CO₂.

Blood samples were allowed to clot at 4 °C. Serum was collected following a 10 min centrifugation at 370 g. The sera were then clarified by a further centrifugation at 400 g for 5 min and the supernatants stored at -20 °C.

2.3.2 CNS Cytosol

Animals were killed by CO₂ inhalation subsequent to cardiac bleeding under halothane/O₂ anaesthesia. The skin was cut away from the skull of each rat and the cranium and spinal column removed to expose the brain and spinal cord. The cerebellum, medulla-pons and cervical spinal cord were removed and cytosolic fractions prepared using the method of Föstermann *et al.* (1990) with minor modifications. Each CNS component was homogenised using a Tri-R Stir-R in 2.5 ml of 0.5 M Tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol and 0.32 M sucrose. Cytosolic fractions were obtained by centrifugation of homogenates at 100,000 g for 1 hr using a Beckman L8-M ultracentrifuge. Contaminating haemoglobin was removed by incubating the samples, 5 % (v/v), with 10 % (w/v) cellulose phosphate (Tris buffer) for 30 min at 4 °C followed by centrifugation at 1000 g for 10 min. Supernatants were transferred to eppendorf tubes and further centrifuged at 1500 g for 2 min. Resulting

supernatants were immediately assayed for nitrite, protein, superoxide and hydrogen peroxide.

2.4 Nitrite Detection

Nitrite, one of the stable end products of NO metabolism, was measured in rat CNS cytosol using an assay developed from that previously described by Green *et al.* (1982). Aliquots of 150 μ l CNS cytosol, standards or blank were added, in triplicate, to a 96 well microtitre plate. Nitrite standards were prepared by dissolving sodium nitrite to 200 μ M in sample buffer and then serially diluting to produce 500 μ l of standards ranging from 0.3 to 200 μ M. An equal volume of Griess reagent consisting of 1 part 1 % (w/v) sulphanilamide in 5 % (v/v) concentrated phosphoric acid, and 1 part 0.1 % (w/v) N-naphthylethylenediamine dihydrochloride was added to each well. Following a 10 min incubation at room temperature absorbance was read at 550 nm using a MR5000 platereader (Dynatech). The nitrite content of each sample was determined from a standard curve obtained by plotting nitrite concentration of standards against absorbance. A typical standard curve prepared in Tris buffer is shown in Figure 2.1. Detection range of the Griess reaction is 1 to 200 μ M.

2.5 Protein Assay

Samples were assayed for protein using the method of Lowry *et al.* (1951). Protein standards were prepared by dissolving BSA at 5 mg/ml in sample buffer and diluting to provide 500 μ l of standard ranging in concentration from 0.0625 to 5 mg/ml. Protein reagent was freshly prepared and consisted of 2 % (w/v) sodium carbonate in

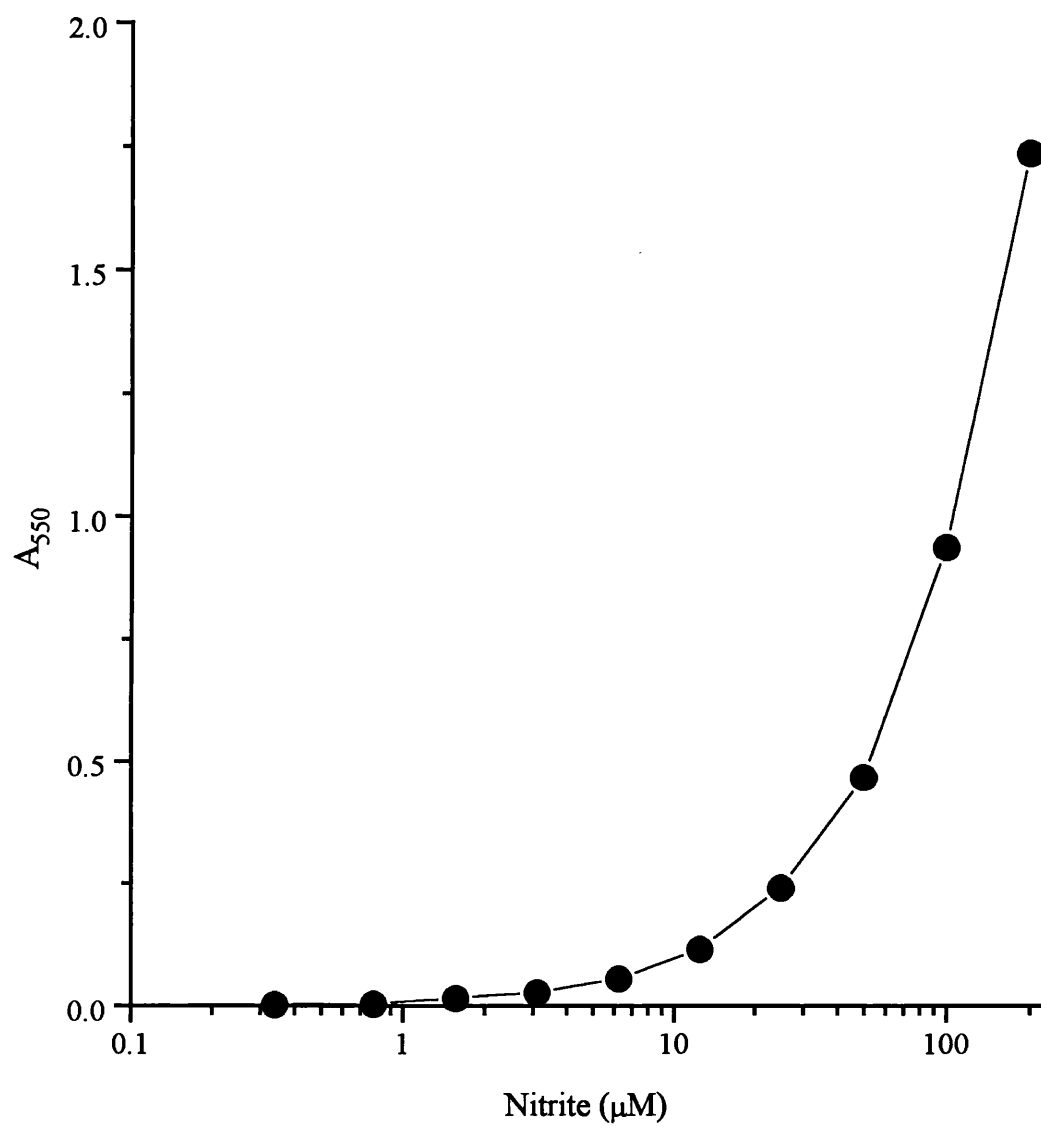


Figure 2.1 Nitrite Standard Curve
A typical curve obtained for the Griess assay.

0.1 M sodium hydroxide, 1 % (w/v) copper sulphate and 2 % (w/v) sodium tartrate in the ratio 100:1:1. Forty microlitre aliquots of sample, standard or blank (appropriate sample buffer) were added, in duplicate, to a 96 well microtitre plate (NUNC) and 200 µl of protein reagent added. After a 10 min incubation at room temperature 10 µl of Folin's Ciocalteu reagent, diluted 1:1 in distilled water immediately prior to use, was added to each well. Absorbance was measured at 595 nm using a MR500 platereader (Dynatech) following a 30 min incubation period. The protein concentration of each sample was calculated from a standard curve obtained by plotting protein concentration of standards against absorbance. A typical standard curve for standards prepared in Tris buffer is shown in Figure 2.2.

2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

The techniques of SDS-PAGE and immunoblotting were undertaken using methods previously established in our laboratory.

2.6.1 Preparation Of Samples And Standards

2.6.1.1 Rat CNS Cytosol Samples

CNS cytosol samples which had been stored at -20 °C were thawed, pooled and their protein content determined as described in section 2.4. Each preparation was then diluted to 0.6 mg/ml or 0.3 mg/ml with Tris buffer for cerebellum samples or medulla-pons and cervical spinal cord samples respectively.

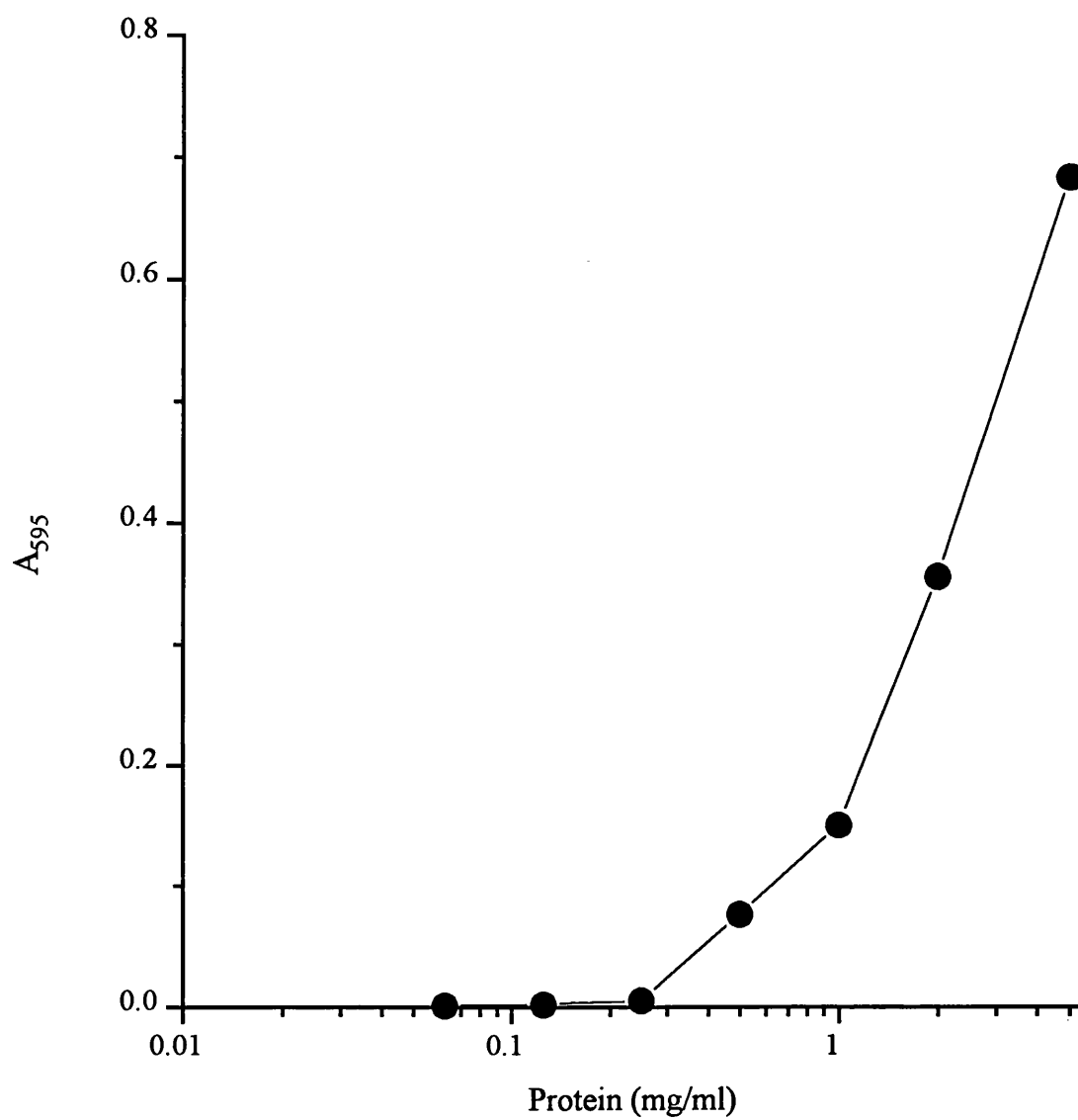


Figure 1.2 Protein Standard Curve

A typical curve for the Lowry protein assay with BSA standards

2.6.1.2 Standards

Rat pituitary tumour cell lysate was used as a positive control for anti-bNOS antibody. The positive control for eNOS antibody comprised of human aortic endothelial cell lysate. Lysate prepared from mouse macrophage cell lines stimulated for 12 hours with IFN- γ (10 ng/ml) and LPS (1 μ g/ml) was utilised as a positive control for the presence of iNOS.

All positive control lysates were provided at a concentration of 0.4 mg protein/ml in SDS-PAGE buffer (62 mM Tris pH 6.8, 2 % (w/v) SDS, 1 % (v/v) β -mercaptoethanol, 0.003 % (w/v) bromophenol blue, 5 % (v/v) glycerol and 1 mM sodium vanadate). Aliquots of control lysates were stored at -20°C.

In each case following the treatment described above the samples or standards were mixed in the ratio 2:1 with reducing sample buffer comprising 0.1 M Tris, 0.1 M N,N-bis(2-hydroxyethyl) glycine (BICINE), 2 % (w/v) sodium dodecyl sulphate (SDS), 5 % (v/v) 2-mercaptoethanol, 10 % (w/v) sucrose and 0.05 % (w/v) bromophenol blue. Samples were heated at 100 °C on a hot block for 5 min and stored at -20°C prior to SDS-PAGE.

2.6.1.3 Molecular Mass Markers

Pre-stained molecular mass markers for immunoblotting (range 4 to 250 kDa) were heated at 100 °C on a hot block for 3 min and stored at 4 °C prior to SDS-PAGE.

2.6.2 SDS-PAGE

SDS-PAGE was performed essentially as described by Laemmli (1970) using a “Mighty Small II” vertical slab gel system (Hoefer Scientific Instruments) according to the manufacturers instructions.

7.5 % acrylamide resolving gels (82 x 60 x 1.5 mm) were prepared from stock solutions of 30 % (w/v) acrylamide/0.8 % (w/v) NN'-methylenebisacrylamide and 1 M

Tris/BICINE. Gels were polymerised chemically by the addition of freshly prepared 10 % (w/v) ammonium persulphate and NNN'N'-tetramethylethylene diamine (TEMED). Final concentrations in the gel were: 7.5 % (w/v) acrylamide, 0.2 % (w/v) NN'-methylenebisacrylamide, 0.1 M Tris, 0.1 M BICINE, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulphate and 0.05 % TEMED. Immediately after pouring, 0.3 ml of 70 % (v/v) ethanol was gently layered onto the surface of each gel. When set the gels were rinsed with distilled water, placed in a sealed polythene bag with some moist filter paper and stored at 4 °C for no longer than one week.

Electrode buffer composed of 0.1 M Tris/BICINE and 0.1 % (w/v) SDS was also prepared from stock solutions. Stacking gels containing 7.5 % (w/v) acrylamide were prepared prior to use by mixing acrylamide/NN'-methylenebisacrylamide stock solution, distilled water and electrode buffer in the ratio 1:1:2. Stacking gels were also polymerised with ammonium persulphate and TEMED as before. The final concentrations in the stacking gels were: 7.5 % (w/v) acrylamide, 0.2 % (w/v) NN'-methylenebisacrylamide, 0.05 M TRIS, 0.05 M BICINE, 0.05 % (w/v) SDS, 0.07 % (w/v) ammonium persulphate and 0.07 % TEMED. Sample wells were formed in the stacking gel by inserting a comb device above the resolving gel. Stacking gel was poured and once set the chamber was filled with electrophoresis buffer. Wells were loaded with 5 - 20 µl of prepared samples or standards using a 25 µl Hamilton syringe.

The gels were run at a constant current of 15 mA/gel until the samples had passed through the stacking gel (approximately 20 min) and then at 35 mA/gel until the bromophenol blue dye front reached the bottom of the gel (approximately 2 hr).

2.6.3 Staining Gels For The Presence Of Protein

Gels were stained for total protein using Coomassie Brilliant Blue. Following a 2 hour incubation with an aqueous solution comprising 0.25 % (w/v) Coomassie Brilliant Blue, 45 % (v/v) methanol and 10 % (v/v) acetic acid, protein was visualised by destaining the background with several changes of a solution containing 20 % (v/v) ethanol and 7.5 % (v/v) acetic acid.

2.6.4 Immunoblotting

Electrophoretic transfer of proteins from unstained gels to polyvinylidene difluoride (PVDF) membrane was performed essentially by the method of Towbin *et al.* (1979) using a “MINI PROTEAN II™” electrophoretic transfer system (Biorad) according to the manufacturers instructions. The electrode buffer was prepared from a stock solution of 0.25 M Tris and 1.92 M glycine and the final solution contained 25 mM Tris, 192 mM glycine and 20 % (v/v) methanol.

Prior to electrophoretic transfer the PVDF membrane was pre-wet in 95 % (v/v) ethanol for 1 min and washed in distilled water for 2-3 min. Finally the membrane was equilibrated in transfer buffer. Proteins were transferred to pre-wet PVDF membranes at a constant voltage of 250 volts for 1 hr.

For the detection of NOS following protein transfer membranes were subjected to the following procedures.

- 1) Incubated with 5 % (w/v) milk powder for 1 hr to block non-specific protein binding sites.
- 2) Washed for 2 x 15 min.
- 3) Probed overnight at 4 °C with primary anti-NOS antibody. See Table 2.2.
- 4) Washed 2 x 15 min.
- 5) Incubated for 1 hr with a 1:1000 dilution of goat anti-mouse IgG.
- 6) Washed 2 x 15 min.
- 7) Incubated with 1:1500 dilution of mouse PAP for 1 hour.
- 8) Washed 1 x 15 min.
- 9) Washed 1 x 15 min in PBS
- 10) Incubated in 0.05 % (w/v) DAB plus 0.02 % (v/v) hydrogen peroxide in PBS until colour developed (approximately 5 to 10 min)
- 11) Washed 2 x 5 min in distilled water.

Table 2.2 Primary antibodies used for immunoblotting

Antibody	Type	Raised Against	Species Specificity	Cross Reactivity	Dilution
Anti-eNOS	Mouse monoclonal	Human endothelial cell NOS	Human, mouse and rat	-	1:5000
Anti-bNOS	Mouse monoclonal	Human bNOS	Human, mouse and rat	iNOS	1:5000
Anti-iNOS	Mouse monoclonal	Mouse macrophage NOS	Human, mouse and rat	-	1:5000

All incubations and washes were carried out at room temperature in PBS containing 0.1 % (v/v) Tween 20 (PBS-Tween) unless otherwise stated. The incubation volume was always 20 ml except for steps using antibodies where it was reduced to 5 ml to conserve reagents.

2.6.5 Comparison Of Immunoblotting Detection Methods

Due to the very low protein concentration of some samples used for immunoblotting various methods were used to enhance band intensity. Several gels were loaded with a range of cervical spinal cord cytosol samples. Immunoblotting was initially performed as described above in section 2.6.4. However, following incubation with the primary antibody detection was carried out using one or a combination of the following methods: peroxidase-anti-peroxidase, nickel and cobalt enhancement and enhanced chemiluminescence (ECL).

2.6.5.1 Peroxidase Anti-Peroxidase Complex

Blots were incubated at room temperature for 1 hour with goat anti-mouse IgG diluted 1: 1000 with PBS-Tween. Subsequently membranes were washed 2 x 15 min in PBS-Tween prior to a 1 hr incubation with a 1:1500 dilution of mouse peroxidase-anti-peroxidase complex. The membrane was again washed in PBS-Tween for 15 min with a further 15 min wash in PBS alone. After this blots were incubated with 0.05 % (w/v) filtered DAB plus 0.02 % (v/v) hydrogen peroxide in PBS until colour developed. Once colour was visible the membranes were rinsed in distilled water for 5 min before being allowed to air dry.

2.6.5.2 Nickel And Cobalt Enhancement

Blots were probed using the techniques described in section 2.6.5.1 followed by incubation with the PAP complex and rinsing in PBS-Tween and PBS, immunoreactive bands were visualised by the following method. A 0.05 % (w/v) solution of DAB was prepared in PBS. An aqueous solution of 1 % (w/v) cobalt chloride and 1 % (w/v)

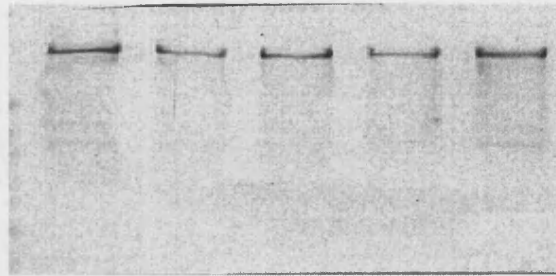
ammonium nickel sulphate (Ni/Co) was prepared and added dropwise to stirring DAB solution with 3 ml of Ni/Co used per 100 ml of DAB. The membranes were incubated in the Ni/Co/DAB solution for 10 min before being transferred to an identical mixture containing 0.02 % (v/v) hydrogen peroxide. Once colour had developed the membranes were washed for 5 min in distilled water and left to air dry.

2.6.5.3 Enhanced Chemiluminescent Detection

Membranes were incubated overnight at 4 °C with primary antibody as before. Following 2 x 15 min washes in PBS-Tween blots, were incubated for 2 hr at room temperature in a 1:5000 dilution of horseradish peroxidase-linked sheep anti-mouse Ig. Blots then underwent two further 15 min washes in PBS-Tween with a final wash in PBS alone for 15 min. An equal volume of Amersham ECL Western blotting reagents 1 and 2 were mixed together to give a final volume equivalent to 0.125 ml/cm² membrane according to the manufacturers instructions. Blots were incubated in ECL reagent for 1 minute before being covered in Saran wrap. Membranes were placed within X-ray exposure cassettes and Kodak X-OMAT film was positioned on top of the blot. Following overnight exposure the film was developed using an RGII X-ray film processor (Fuji).

The combination of PAP complex with Ni/Co enhancement and ECL detection were found to give maximum sensitivity with acceptable background staining (Figure 2.3). For titration of reagents used in the PAP method a series of blots containing identical CNS cytosol samples were prepared. After probing with the primary antibody each blot was divided into strips which were incubated with a range of concentrations of the secondary reagent (goat anti-mouse IgG) and then developed in the normal way. Similarly the second blot was used to titrate the tertiary reagent (PAP complex). Optimum dilutions of goat anti-mouse IgG and PAP were 1:1000 and 1:1500 respectively (Figure 2.4).

(A)



(B)



(C)

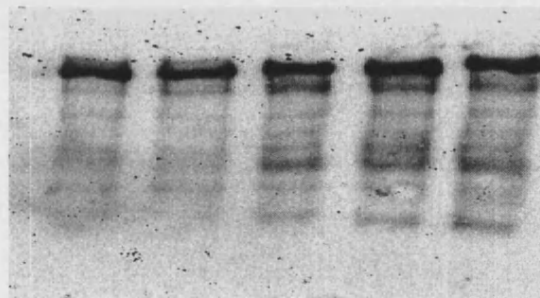


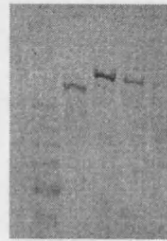
Figure 2.3 Comparison of immunoblotting detection methods

(A) PAP

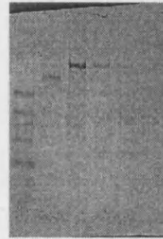
(B) PAP with Ni/Co enhancement

(C) ECL

(A) NOS Primary Antibody



1:2500



1:5000

(B) Goat anti-mouse IgG



1:250

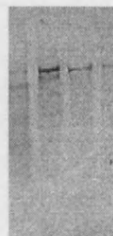


1:750



1:1000

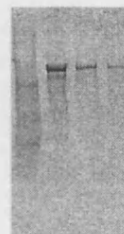
(C) PAP



1:500



1:1000



1:1500

Figure 2.4 Titration of antibody reagents for immunoblotting

(A) Anti-bNOS antibody, optimum dilution 1:5000

(B) Goat anti-mouse IgG, optimum dilution 1:1000

(C) PAP, optimum dilution 1:1500

To check the specificity of staining, control blots containing representative CNS cytosol samples were subjected to the same immunoblotting procedures except that the primary antibody was omitted and replaced with PBS.

2.6.6 Estimation Of Molecular Mass

To estimate molecular mass of unknown bands on gels and blots, Rf values were calculated for standards and unknowns using the formula:

$$\begin{array}{l} \text{Rf} \\ \text{(Ratio of fronts)} \end{array} = \frac{\text{distance moved by band (mm)}}{\text{distance moved by dye front (mm)}}$$

The Rf values of standards were plotted against molecular mass and the calibration curve used to calculate the molecular mass of unknowns. A typical standard curve for molecular mass markers is shown in Figure 2.5.

2.6.7 Densitometry Of Immunoblots

In order to provide a semi-quantitative assessment of NOS levels, the intensity of immunoreactive bands was measured using a GS-670 imaging densitometer (Biorad) and Molecular Analyst software (Biorad). Results were expressed as % change from normal values.

2.7 Superoxide Production

The production of superoxide in CNS cytosol samples was determined using the reduction of ferricytochrome C. One hundred microlitre aliquots of CNS cytosol sample or Tris buffer blank were added, in quadruplicate, to a 96 well microtitre plate. Samples and blank were diluted by the addition of 50 μ l of 1mM HBSS with 1 mM $\text{Ca}^{2+}/\text{Mg}^{2+}$. Ferricytochrome C was prepared in 1mM HBSS with 1 mM $\text{Ca}^{2+}/\text{Mg}^{2+}$ to give a final concentration of 320 μ M. Each well received 50 μ l of this mixture and the absorbance of

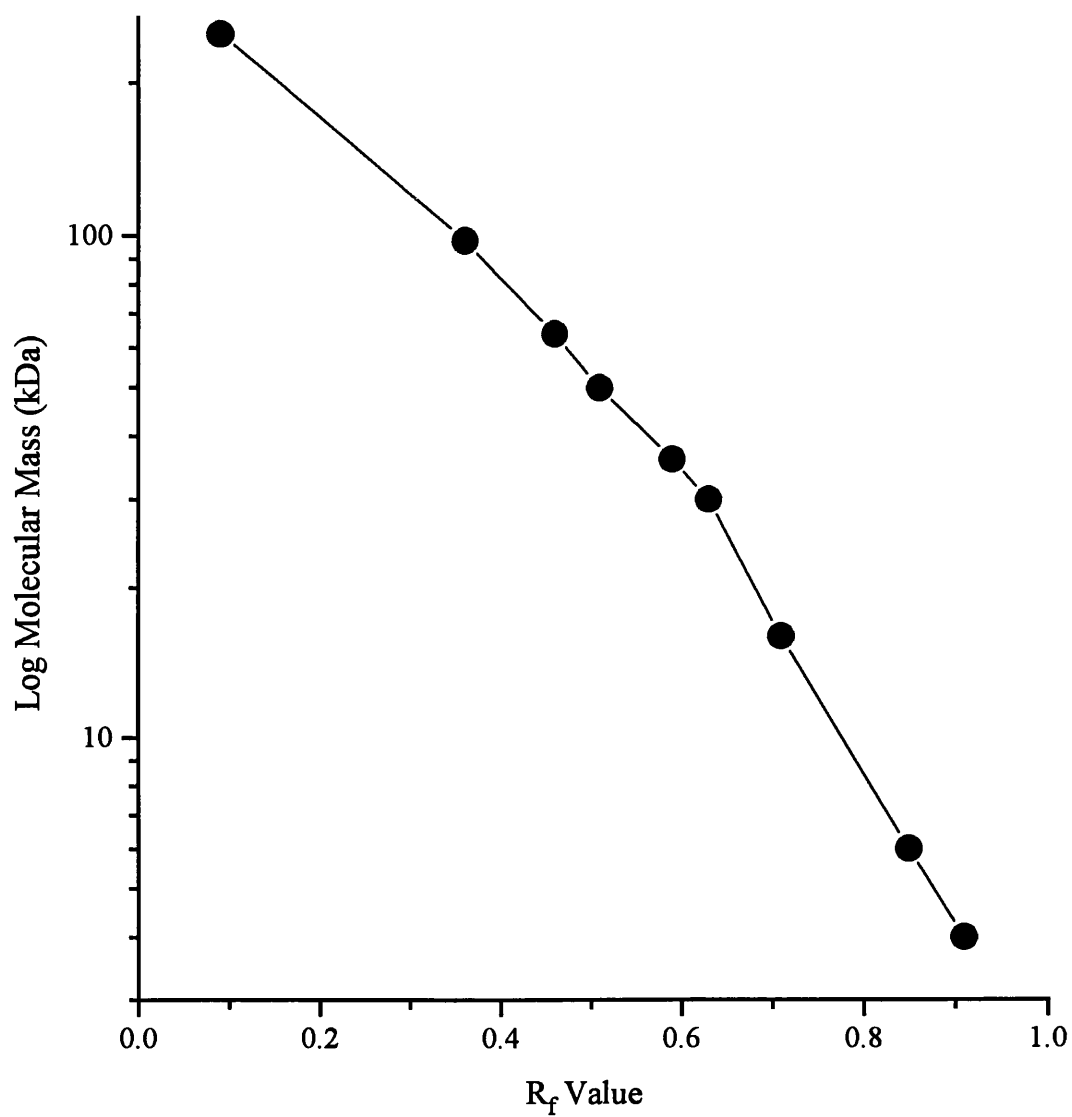


Figure 2.5 A representative standard curve of migration of protein standard on SDS-gel obtained using pre-stained molecular weight markers.

the plate at 550 nm was determined every 5 min over a one hr period. The background values of the Tris buffer blank were subtracted from those of the samples and the amount of superoxide present per well at T₃ was calculated from the extinction coefficient for the absorption of reduced ferricytochrome C, minus oxidised cytochrome C as read at 550 nm, by the formula $E_{550\text{nm}} = 21 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ (Massey 1959). As the length of the light path passing through the cytochrome C solution added to the wells was less than 1 cm, a correction factor was introduced based on the fact that the measured length of the vertical light path is 6 mm, when 200 µl volumes per well are dispensed in 96 well plates.

With this correction it was calculated that the amount of superoxide produced per well is expressed by the formula :

$$\text{n moles superoxide} = \frac{\text{O.D.} \times 200}{12}$$

The superoxide of each sample was corrected for protein content and expressed as n moles superoxide/mg protein.

The specificity of the reaction was determined by the addition of 250 U/ml superoxide dismutase (SOD) to CNS cytosol samples. Following addition of SOD no ferricytochrome C reduction was observed as spectrophotometric readings for CNS cytosol samples in the presence of SOD were not above background levels. This confirms that differences in optical density were due to the production of superoxide.

2.8 Hydrogen Peroxide Content

The level of hydrogen peroxide within CNS cytosol preparations was ascertained by measuring the oxidation of scopoletin to give a non-fluorescent product. Ten microlitre aliquots of CNS cytosol or hydrogen peroxide standard were added in triplicate to 96 well Fluoroplate (Titertek). Standards were prepared by diluting hydrogen peroxide in CNS cytosol buffer to give a concentration of 10 nM. A series of standards ranging from 0.5 to 6 nM were then obtained by diluting the 10 nM stock solution. Standards and

samples then underwent a further dilution by the addition of 150 µl of distilled water. Ten microlitres of a reaction mixture composed of 138 µg/ml scopoletin, 15 mM sodium azide, 1 mg/ml horseradish peroxidase, 2 mM Ca²⁺ and 2 mM Mg²⁺ was added to each well.

Fluorescence of the samples and standards was measured every 5 minutes for a 30 minute period using a Titretek Fluoroscan II with excitation at 355 nm and emission at 460 nm. The hydrogen peroxide content of each sample at T3 was determined by fitting an exponential curve equation (2.1) to the standard hydrogen peroxide values

$$y = y_0 + Ae^{-\left(\frac{x-x_0}{t}\right)} \quad (2.1)$$

$$e^{-\left(\frac{x-x_0}{t}\right)} = \frac{y-y_0}{A} \quad (2.2)$$

$$-\frac{x-x_0}{t} = \ln\left(\frac{y-y_0}{A}\right) \quad (2.3)$$

$$x = \left(-\ln\left(\frac{y-y_0}{A}\right)\right) \cdot t - x_0 \quad (2.4)$$

Rearranging this equation and as $x_0=0$ equation 2.4 becomes:

$$x = \left(-\ln\left(\frac{y-y_0}{A}\right)\right) \cdot t \quad (2.5)$$

where x = nmol of hydrogen peroxide produced; y = remaining fluorescence after the cells have released hydrogen peroxide; y_0 , A and t are constants determined when fitting the standard curve. A typical standard curve is shown in Figure 2.6. The spreadsheet Excel (version 5) was used to manipulate the data and calculate the hydrogen peroxide produced from the changes in fluorescence.

Scopoletin oxidation by hydrogen peroxide in CNS samples was confirmed by the addition of 250 U/ml catalase to CNS cytosols prior to the assay. In the presence of

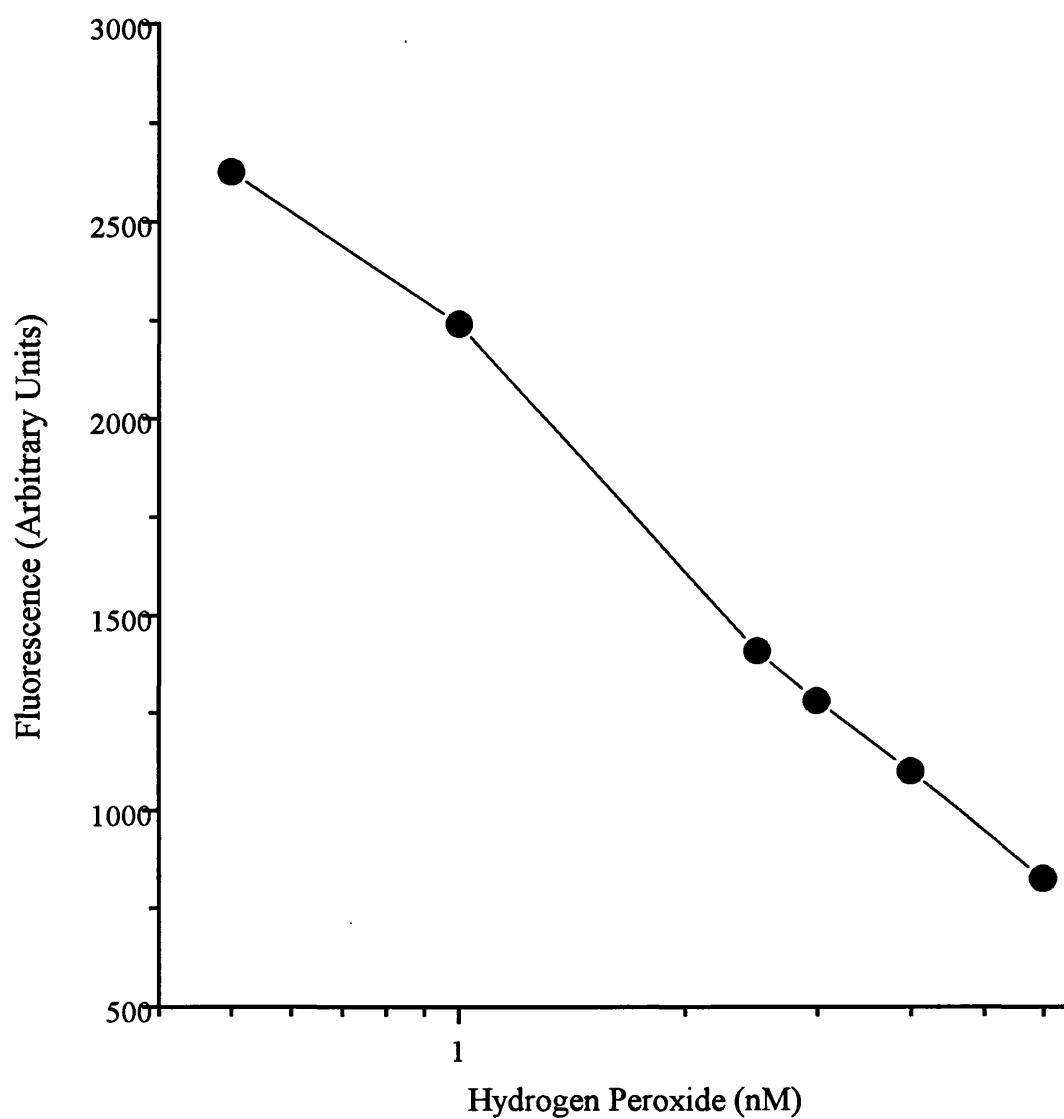


Figure 2.6 Hydrogen Peroxide Standard Curve

A representative curve obtained for the scopoletin assay of hydrogen peroxide with standards prepared in CNS cytosol buffer.

catalase no oxidation of scopoletin was observed thus indicating that the reaction is specific for determining hydrogen peroxide levels in CNS cytosol.

2.9 Histology

Dissected cervical spinal cord tissues were wrapped in aluminium foil and frozen on dry ice. Samples were stored at -80 °C prior to sectioning. Cervical spinal cord sections, 20 micron thick, were cut at -18 °C using a Bright cryostat, mounted onto glass slides coated with 1 % gelatin and 0.1 % chromallum and stored at -80 °C until stained.

Sections were fixed prior to staining by immersing in acetone for 5 min. Air dried sections were stained in haematoxylin and eosin. Cervical spinal tissues were stained in Meyer's haematoxylin for 3 minutes. The sections were then washed thoroughly in tap water for 5 minutes before staining with eosin for 3 minutes. A further 5 minute wash in running tap water was undertaken to allow differentiation. Slides were then dehydrated through graded alcohol and xylene before being mounted in DPX. The number of perivascular infiltrates per section was determined by light microscopy.

2.10 Radioimmunoassay for Corticosterone

Corticosterone in rat serum was measured using an ICN RSL ¹²⁵I-corticosterone radioimmunoassay (RIA) kit obtained from IDS. The basis of this assay is that the corticosterone present in an unknown serum sample competes with a known quantity of ¹²⁵I-labelled corticosterone to bind a limited amount of specific antibody. Thus the more corticosterone that is present in the sample, the less radiolabelled corticosterone is able to bind to the antibody. The antigen-antibody complexes formed are separated from unbound corticosterone by precipitation and centrifugation and the amount of

radiolabelled corticosterone complexed with the antibody is estimated by gamma-counting the pellet. The radioactive counts are used to calculate the percentage of ^{125}I -corticosterone bound, which is inversely proportional to the amount of corticosterone in the sample.

The RIA has a range of 0.39 - 62 ng/ml and was performed according to the manufacturer's instructions, except that serum samples were assayed at a 1:100 dilution instead of the recommended 1:20. To reduce the influence of assay to assay variation serum samples from the same experiment were run in the same RIA.

2.10.1 Assay Protocol

The assay was performed in duplicate in 12 x 75 mm borosilicate disposable glass tubes.

- 1) Rat serum was thawed and diluted 1:100 with steroid assay buffer.
- 2) 200 μl steroid assay buffer was added to non-specific binding (NSB) tubes and 100 μl to zero calibrator tubes (Bo).
- 3) 100 μl of corticosterone standards and diluted rat serum samples were added to appropriate tubes.
- 4) 100 μl of ^{125}I -corticosterone was added to all tubes.
- 5) 100 μl of anti-corticosterone was added to all tubes except NSB.
- 6) Tubes vortexed and incubated at 4 °C overnight.
- 7) 100 μl of secondary precipitation antibody was added to all tubes.
- 8) Tubes vortexed and incubated at room temperature for 1 hr.
- 9) All tubes centrifuged at 1500 g for 15 min.
- 10) Supernatant in each tube was decanted, to leave pellet, and the rim of the tube was blotted dry on absorbent paper.
- 11) Precipitates were counted in a LKB 1277 Gammamaster automatic gamma counter for 2 minutes.

2.10.2 Calculations

Results were expressed in counts per minute (CPM) and the percentage ^{125}I -corticosterone bound was calculated for both standards and samples using the formula:

$$\% \text{ B/Bo} = \frac{\text{Mean CPM (sample)} - \text{Mean CPM (NSB)}}{\text{Mean CPM (Bo)} - \text{Mean CPM (NSB)}} \times 100$$

Where: $\% \text{ B/Bo}$ = percentage ^{125}I -corticosterone bound

Mean CPM (sample) = mean CPM of standard or sample

Mean CPM (NSB) = mean CPM of non-specific binding

Mean CPM (Bo) = mean CPM of standard containing 0
ng/ml corticosterone i.e. 100 % binding

A standard curve of percentage bound against log concentration was constructed and the corticosterone concentration of unknown samples read directly from the curve. A typical curve is shown in Figure 2.7. Values obtained for samples which had been diluted 1:100 were corrected by multiplying by 100.

2.11 Statistical Analysis

All results were expressed as mean values \pm S.E.M., of n observations, except for Western blotting data which were denoted as a % change from the normal value.

Evaluation of significant differences in neurological disease parameters such as body weight changes, day of disease onset, peak disease severity and cellular infiltration, as well as in CNS levels of nitrite, superoxide and hydrogen peroxide between each group were performed using the Kruskal-Wallis test with post-hoc Mann Whitney U test and Bonferroni correction for multiple comparisons. The incidence of neurological disease and paralytic symptoms was analysed by the Chi-squared test. In all tests $p < 0.05$ was considered significant.

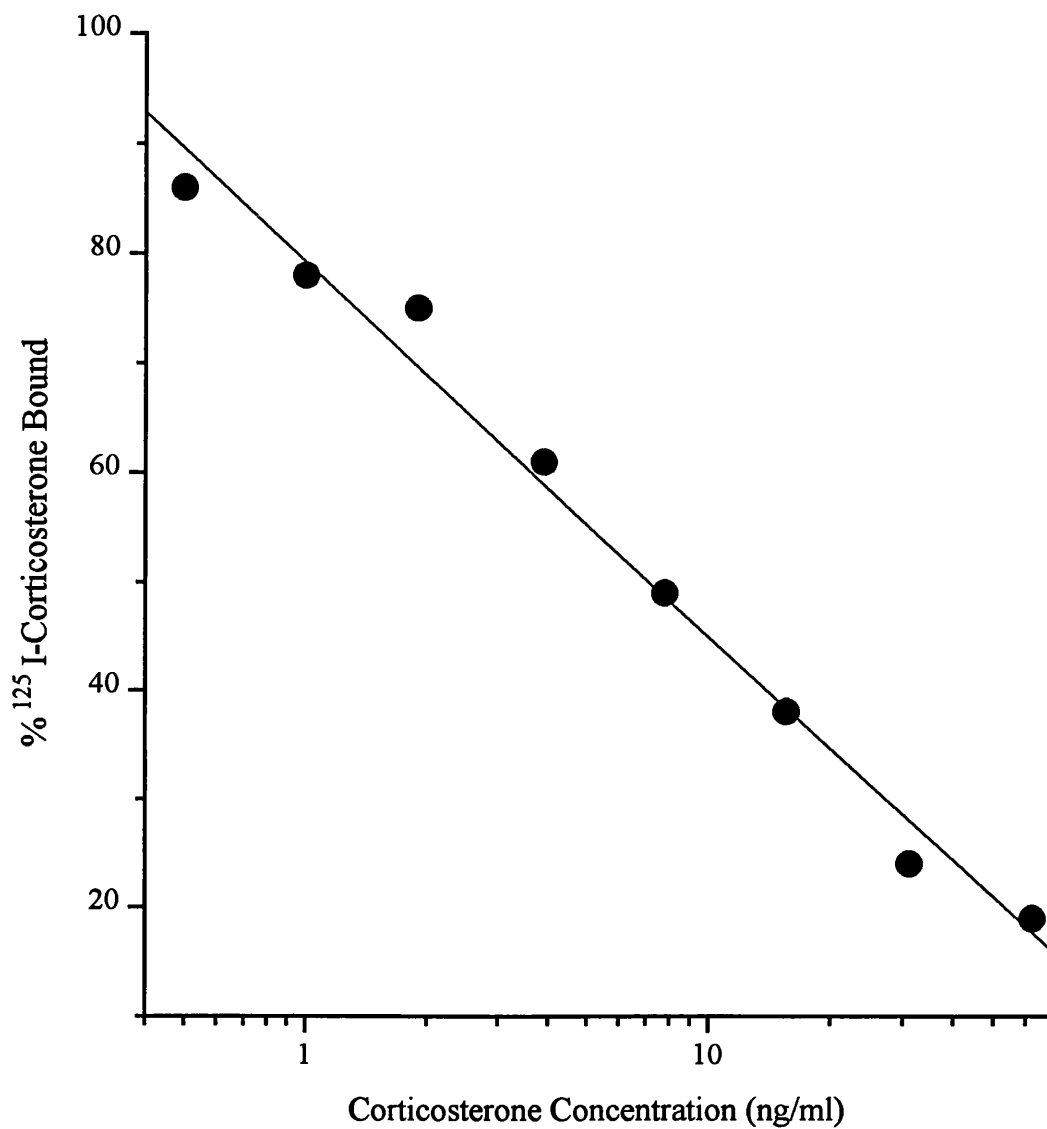


Figure 2.7 Corticosterone Standard Curve

A representative standard curve obtained using the ICN ^{125}I -corticosterone radioimmunoassay kit.

CHAPTER 3

QUANTITATION OF NO

IN EAE

3. RESULTS

3.1 Characterisation of Actively Induced EAE

Following inoculation of an encephalitogenic emulsion Lewis rats developed an acute monophasic paralytic disease which was self-limiting. Lewis rats sensitised for EAE developed disease signs within 11 to 14 days of immunisation. Initially a sudden and dramatic weight loss was observed (day 10 to 11 P.I.) which continued until the beginning of the recovery phase (day 14 to 15 P.I.) (Figure 3.1a). Weight loss was proceeded by the development of ascending paralysis, the extent of which was determined using a 4 point severity scale (Figure 3.1b). Disease severity peaked at around day 13 P.I. with recovery from neurological signs occurring from approximately day 14 P.I. onwards (Figure 3.1b). The recovery phase was complete by about day 22 P.I. (Figure 3.1b) with animals continuing to gain weight and display no signs of disease until the study ended on day 28 P.I. (Figure 3.1).

3.2 Nitrite Levels in CNS Cytosol

Although a role for NO in EAE has been demonstrated by other workers (MacMicking *et al.* 1992; Koprowski *et al.* 1993; Lin *et al.* 1993) *ex vivo* measurements of NO in CNS tissues from EAE-diseased animals have not been carried out. Therefore nitrite levels were determined in CNS cytosol fractions from EAE-inoculated Lewis rats as an indicator of NO production and possible role in disease pathogenesis. Studies by us have shown that at day 13 P.I. EAE-sensitised rats display peak disease severity (Figure 3.1) both CFA-inoculated and EAE-diseased animals were sampled on this day.

Nitrite content was measured in the cerebellum, medulla-pons and cervical spinal cord regions of the CNS as these areas are differentially affected by EAE with respect to inflammatory cell infiltrates and BBB permeability (Bolton *et al.* 1984, Paul & Bolton 1995). Levels of nitrite in the medulla-pons and cervical spinal cords from normal animals were in excess of values recorded in the cerebellum tissue (cerebellum 128 ± 11 ,

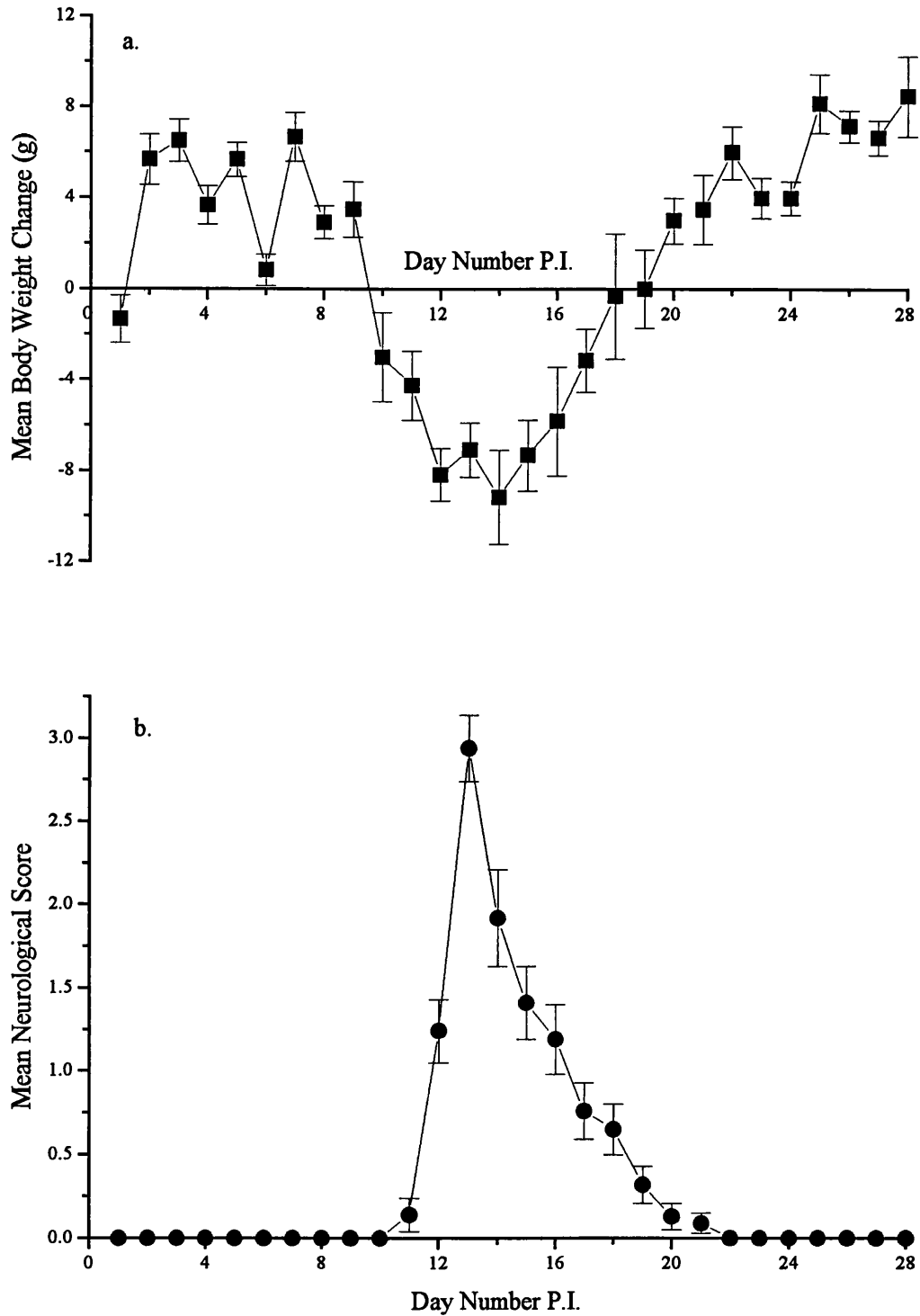


Figure 3.1 Characterisation of actively induced EAE.

EAE was induced in Lewis rats following inoculation with an encephalitogenic emulsion. Animals were assessed daily for changes in body weight (a) and neurological signs of disease (b). Body weight changes were expressed as mean body weight change (g) \pm S.E.M., $n = 8$. Neurological disease symptoms were scored on a severity scale from 1 to 4 and results are expressed as mean neurological score \pm S.E.M., $n = 16$.

medulla-pons 178 ± 26 , cervical spinal cord 202 ± 25 μ moles nitrite/mg protein \pm S.E.M., $n=12$) (Figure 3.2). Nitrite content in CNS tissues from CFA-inoculated rats were comparable to values recorded in normal rats (cerebellum 114 ± 17 , medulla-pons 283 ± 28 , cervical spinal cord 247 ± 15 , $n=10$) (Figure 3.2). In contrast established neurological EAE, coinciding with peak disease signs, was characterised by a significant increase in the nitrite content of all isolated CNS preparations (cerebellum 319 ± 45 , $p<0.001$; medulla-pons 481 ± 65 , $p<0.001$; cervical spinal cord 313 ± 25 , $p<0.001$, $n=12$) (Figure 3.2). Furthermore nitrite levels in CNS tissues from EAE-diseased animals were significantly elevated compared to values obtained in CFA-inoculated rats (cerebellum $p<0.001$, medulla-pons $p<0.01$, cervical spinal cord $p<0.01$).

3.3 EAE Profile of Nitrite Levels in CNS Cytosol

Following the determination of a disease specific increase in CNS cytosol nitrite levels in EAE-sensitised animals the possibility of a relationship between disease development and CNS nitrite levels was examined. EAE-inoculated Lewis rats were sacrificed at various timepoints throughout the disease and CNS cytosol nitrite content determined (Figure 3.3).

In cerebellar tissue a direct correlation between CNS cytosol nitrite content and disease severity was identified (Figure 3.3a). Nitrite levels in cerebellar tissues of EAE-sensitised animals sacrificed 7 days P.I. were elevated compared to normal values (day 7 P.I. 185 ± 47 vs normal 128 ± 11 μ moles nitrite/mg protein \pm S.E.M., $n= 8$ and 12 respectively). The increase in nitrite content continued as the disease progressed (weight loss 234 ± 43 , $p<0.05$, $n=7$) with maximal levels obtained when animals displayed peak disease symptoms (height of disease 337 ± 45 , $p<0.001$, $n=12$) (Figure 3.3a). Interestingly, nitrite levels in the tissue remained significantly elevated during recovery (recovery 269 ± 26 , $p<0.001$, $n=9$) (Figure 3.3a). However, by 28 days P.I. cerebellar cytosol nitrite content had decreased to within normal limits (day 28 P.I. 115 ± 35 , $n=8$) (Figure 3.3a).

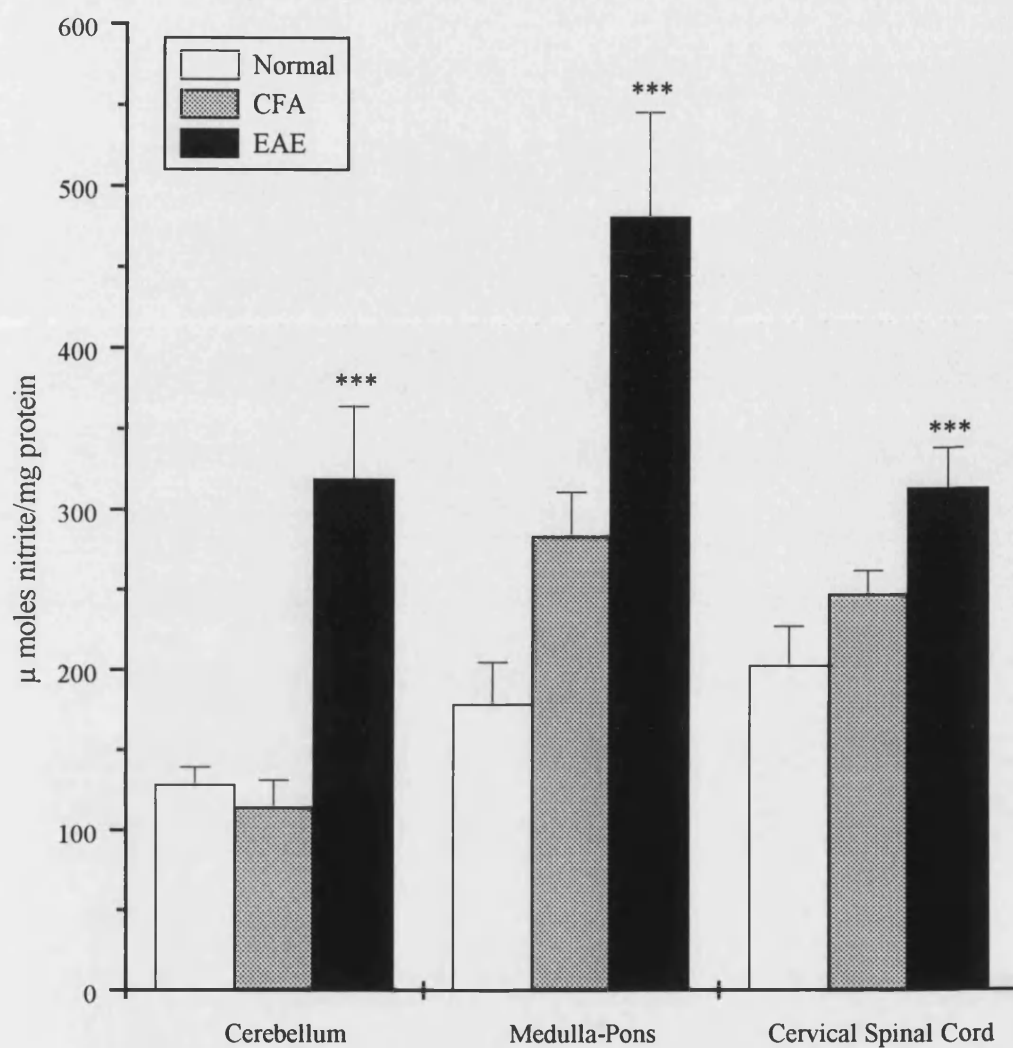


Figure 3.2 Nitrite levels in CNS cytosol.

CNS cytosol nitrite levels were measured using the Griess assay and following protein content determination results were expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, $n = 12$; CFA control inoculated Lewis rats, sampled on day 13 P.I., $n = 10$ and EAE-diseased Lewis rats, sampled on day 13P.I., $n = 12$. *** $p < 0.001$ Mann Whitney U test compared to normal animals.

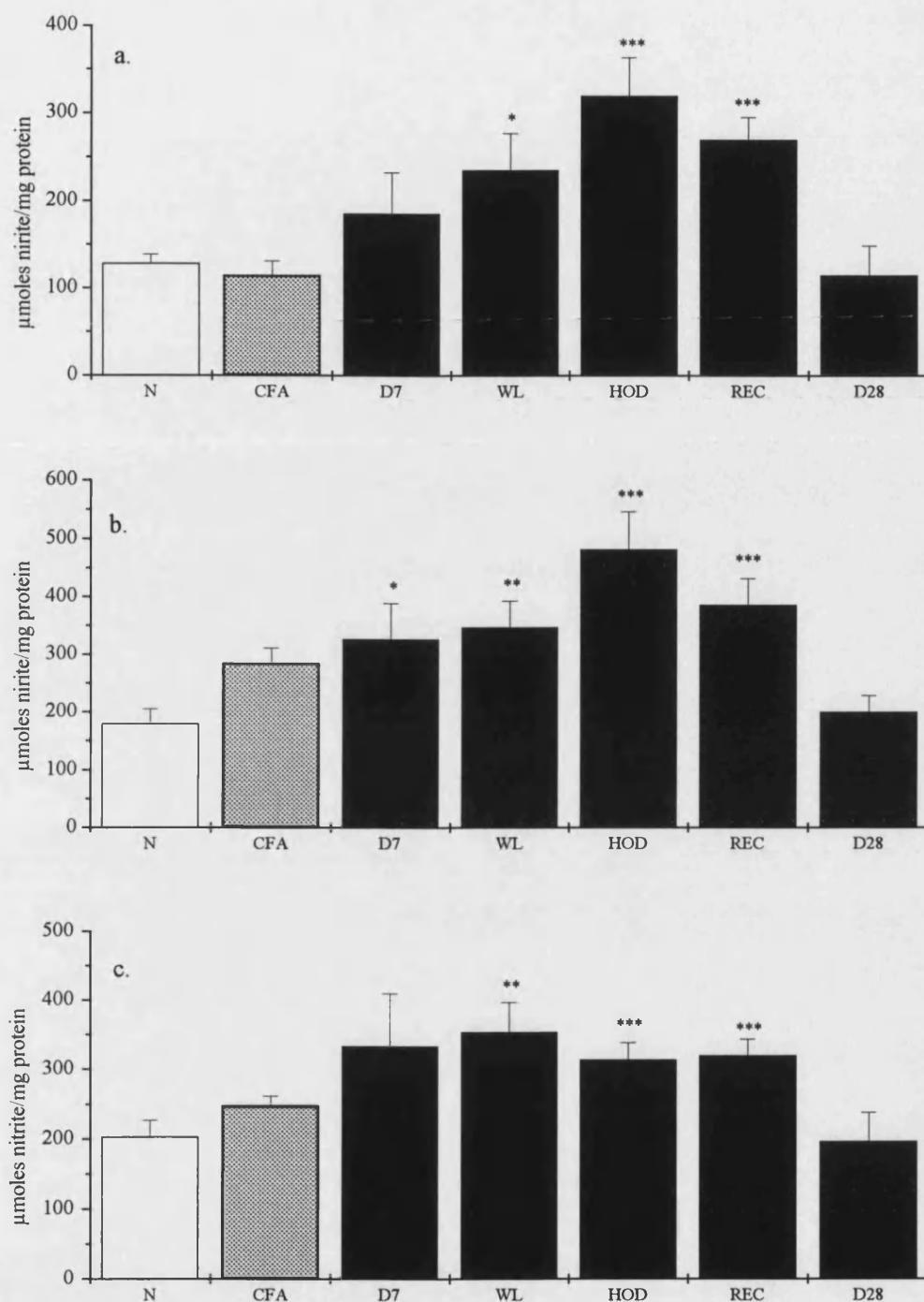


Figure 3.3 EAE profile of nitrite levels in CNS cytosol.

CNS cytosol was prepared from the a. cerebellum, b. medulla-pons and c. cervical spinal cord areas of each animal. Nitrite levels were measured by the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats (N), n = 12; CFA inoculated rats, sampled day 13 P.I., n = 10 and EAE-sensitized Lewis rats sampled at day 7 P.I. (D7), n = 8; weight loss (WL), n = 7; height of disease (HOD), n = 12; recovery (REC), n = 9 and day 28 P.I. (D28), n = 8. *p<0.05, **p<0.01, ***p<0.001 Mann Whitney U test compared to normal animals.

A similar profile of nitrite levels was recorded in CNS cytosol extracts from the medulla-pons of EAE-inoculated rats (Figure 3.3b). Medulla-pons cytosol content increased as neurological deficits of EAE developed (normal 178 ± 26 , $n=12$; day 7 P.I. 326 ± 63 , $p<0.05$, $n=8$; weight loss 346 ± 47 , $p<0.01$, $n=7$) with maximal levels attained when peak signs of disease were demonstrated (height of disease 481 ± 65 , $p<0.001$, $n=12$) (Figure 3.3b). Although the nitrite content of medulla-pons cytosol was significantly enhanced during recovery (recovery 384 ± 47 , $p<0.001$, $n=9$), levels had returned to normal by 28 days P.I. (day 28 P.I. 199 ± 30 , $n=8$) (Figure 3.3b).

By comparison there was no correlation between EAE disease progression and cervical spinal cord cytosol nitrite content (Figure 3.3c). Levels of nitrite in cervical spinal cord cytosol fractions were elevated throughout the induction and active phases of EAE with the maximum value recorded when animals displayed weight loss (normal 202 ± 25 , $n=12$; day 7 P.I. 332 ± 78 , $n=8$; weight loss 353 ± 44 , $p<0.01$, $n=7$; height of disease 313 ± 25 , $p<0.001$, $n=12$) (Figure 3.3c). During the recovery phase of the disease the nitrite content of the cervical spinal cord remained increased (319 ± 24 , $p<0.001$, $n=9$). However by day 28 P.I. cervical spinal cord cytosol nitrite levels were comparable to values recorded in normal animals (day 28 P.I. 196 ± 43 , $n=8$) (Figure 3.3c).

3.4 EAE Disease Profile of NOS Expression

Although nitrite levels were elevated in the CNS cytosol samples from EAE-inoculated animals the mechanisms responsible for the enhancement are unknown. Increases in nitrite levels may arise as a result of induction of NOS enzymes. Therefore, cerebellar, medulla-pons and cervical spinal cord cytosol preparations were assessed individually for the presence of the 3 NOS enzymes, eNOS, bNOS and iNOS, using semi-quantitative densitometric analysis. Following separation on SDS-gels, CNS cytosol samples contained immunospecific bands at approximately 140 kDa, 155 kDa and 135 kDa subsequent to immunoblotting with anti-eNOS, anti-bNOS and anti-iNOS antibodies respectively (Figure 3.4) which corresponds to the molecular weights of the NOS enzymes.

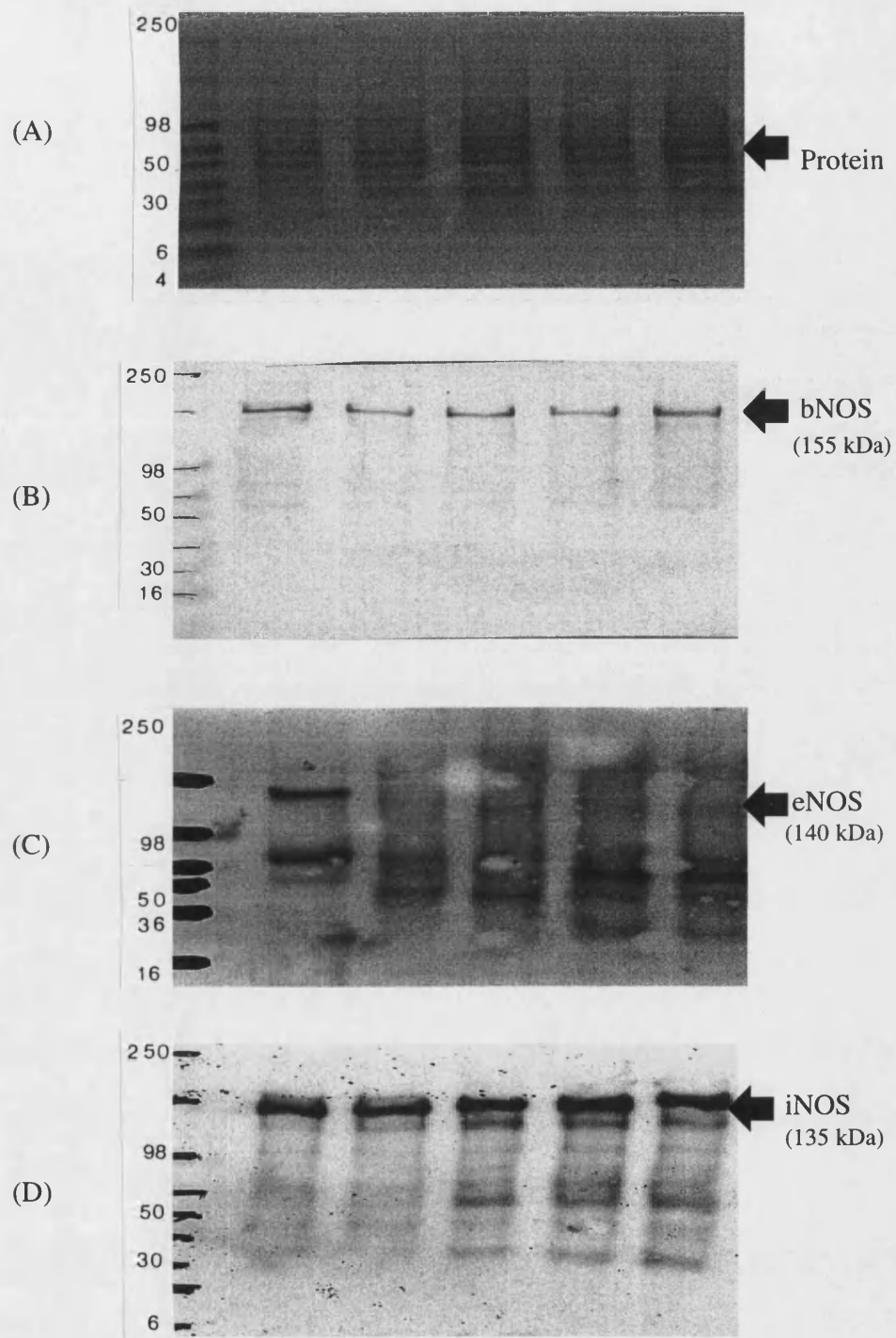


Figure 3.4 SDS-PAGE and Western blotting of CNS cytosol

CNS cytosol proteins were separated on SDS-gels (A) and following electrophoretic transfer they were Western blotted with anti-bNOS (B), anti-eNOS (C) and anti-iNOS (D) antibodies.

3.4.1 EAE Disease Profile of Cerebellum NOS Protein Expression

Normal animals exhibited basal levels of eNOS, bNOS and iNOS in their cerebellar cytosol (Figure 3.5). Moreover, animals sensitised for EAE contained elevated amounts of all NOS proteins in their cerebellar tissue. However, the profile of expression differed for each isoform of the enzyme (Figure 3.5).

Expression of eNOS was increased during active EAE with maximal protein levels obtained at day 7 P.I. (139 % above normal) (Figure 3.5a). eNOS levels then decreased as disease signs developed, with minimum values occurring when animals exhibited peak disease severity (35 % above normal) (Figure 3.5a). Interestingly, eNOS expression was again elevated during the recovery phase (121 % above normal) although by day 28 P.I. the protein levels had returned to normal (3 % above normal) (Figure 3.5a). Furthermore, cerebellar cytosol preparations from CFA-inoculated rats also contained increased levels of eNOS enzyme (85 % above normal) (Figure 3.5a).

bNOS expression in cerebellum samples from CFA control animals was not markedly different to that of normal animals (16 % above normal) (Figure 3.5b). In contrast, throughout the active phase of EAE bNOS enzyme levels were elevated, with maximal and minimal expression produced at peak disease severity and weight loss respectively (height of disease 159 %; weight loss 57 % above normal) (Figure 3.5b). Moreover, during disease recovery bNOS expression remained enhanced (92 % above normal). However by day 28 P.I. bNOS protein levels had returned to CFA control levels (17 % above normal) (Figure 3.5b).

In cerebellar cytosol fractions of EAE-sensitised Lewis rats a direct correlation between iNOS expression and disease development was identified (Figure 3.5c). Levels of iNOS protein in the cerebellum of EAE-inoculated animals sacrificed at day 7 P.I. were slightly increased compared to normal values (15 % above normal) (Figure 3.5c). The elevation in iNOS expression continued as the disease progressed, with maximal levels detected at the height of disease (weight loss 52 %; height of disease 245 % above normal) (Figure 3.5c). iNOS protein expression was still increased during recovery and even at day 28 P.I. (recovery 48 %, day 28 P.I. 21 % above normal) (Figure 3.5c). In

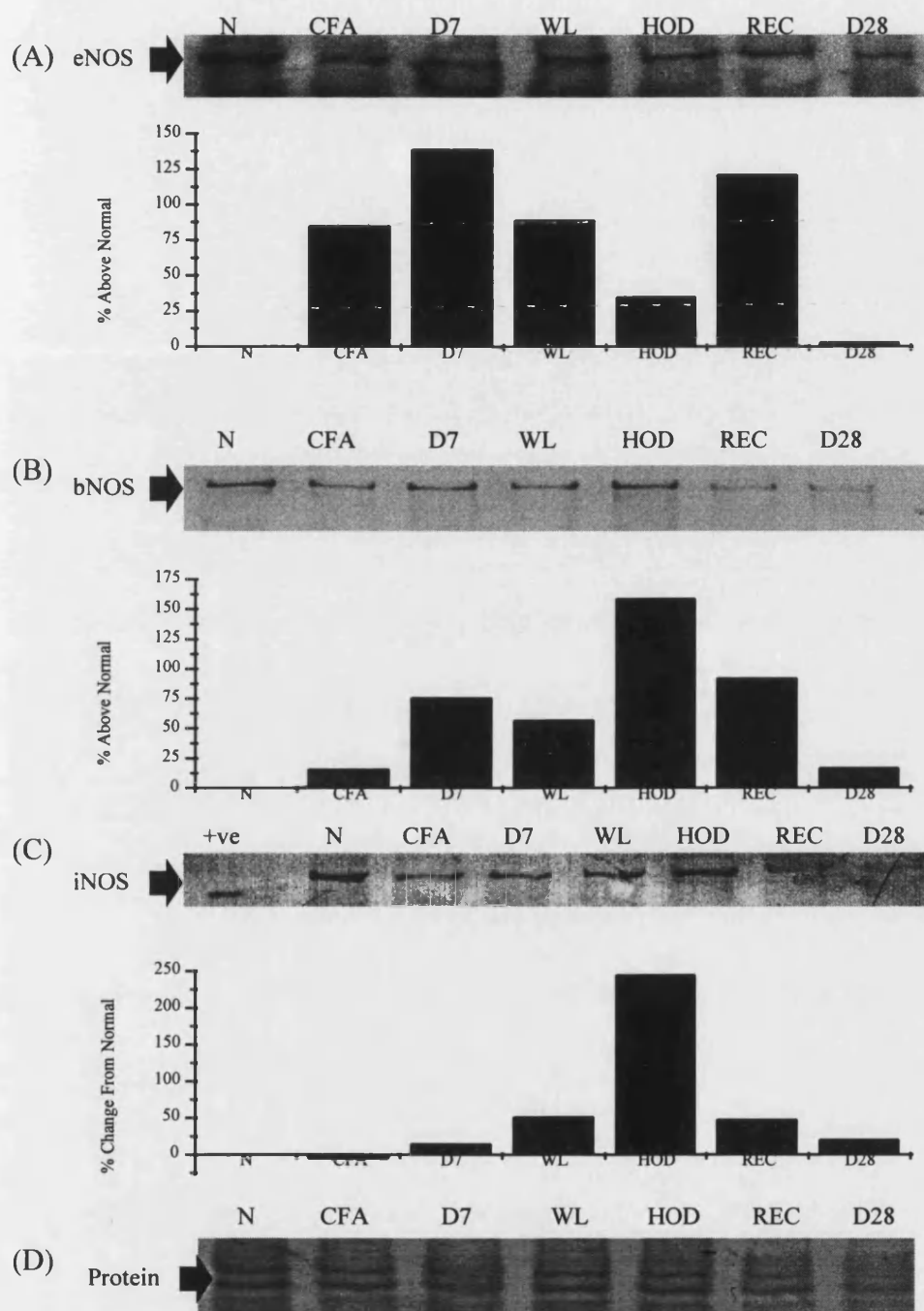


Figure 3.5 EAE disease profile of cerebellar NOS protein expression

CNS cytosol was obtained and pooled from the cerebellum of 3 normal Lewis rats (N); CFA-inoculated Lewis rats (CFA) and EAE-inoculated animals at day 7 P.I. (D7); weight loss (WL); height of disease (HOD); recovery (REC) and day 28 P.I. (D28). CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with (A) anti-eNOS; (B) anti-bNOS and (C) anti-iNOS antibodies. NOS protein was detected using ECL (A) or PAP complex (B & C). Densitometric analysis of blots and SDS-gels (D) was undertaken and following correction for differences in total protein content NOS levels were expressed as % change from normal value. Results shown are representative of 3 separate experiments.

contrast no marked differences were observed in iNOS protein content of CFA-inoculated and normal rats cerebellar cytosol (CFA 7 % below normal) (Figure 3.5c).

EAE-diseased animals displayed elevated levels of all 3 NOS-isoforms in their cerebellum. Therefore, the increased nitrite production observed in this CNS area (Figure 3.2 and 3.3) may not be attributed solely to enhancement of a single NOS enzyme but from the combined effects of all isoforms.

3.4.2 EAE Disease Profile of Medulla-Pons NOS Protein Expression

Basal levels of eNOS, bNOS and iNOS protein were identified in medulla-pons cytosol preparations from normal Lewis rats (Figure 3.6). By comparison, EAE-inoculated animals expressed increased amounts of the 3 NOS isoforms in medulla-pons tissue. However the profile of enzyme expression in EAE-diseased rats was different for each isoform (Figure 3.6).

eNOS expression in medulla-pons tissue was slightly increased in EAE-sensitised animals at day 7 P.I. (13 % above normal). However, eNOS protein in medulla-pons cytosol had decreased to normal levels when animals displayed weight loss (5 % above normal) (Figure 3.6a). Thereafter eNOS expression in EAE-diseased Lewis rats was elevated with maximal values obtained when animals had recovered (44 % above normal) although similar levels of eNOS protein were present in medulla-pons tissue from CFA-inoculated rats (30 % above normal) (Figure 3.6a). By day 28 P.I., eNOS expression had fallen to below normal levels (27 % below normal) (Figure 3.6a).

No correlation was observed between EAE-disease development and bNOS expression in medulla-pons cytosol preparations (Figure 3.6b). Indeed, although bNOS levels were elevated during EAE maximal levels were obtained prior to the onset of neurological disease signs (day 7 P.I. 25 %, weight loss 58 % above normal) (Figure 3.6b). During recovery bNOS expression was similar to that attained at peak disease severity (height of disease 19 %, recovery 16 % above normal). However following the loss of neurological deficits 28 days P.I. bNOS protein levels had decreased to just below the value obtained in normal animals (3 % below normal). In addition, CFA-control

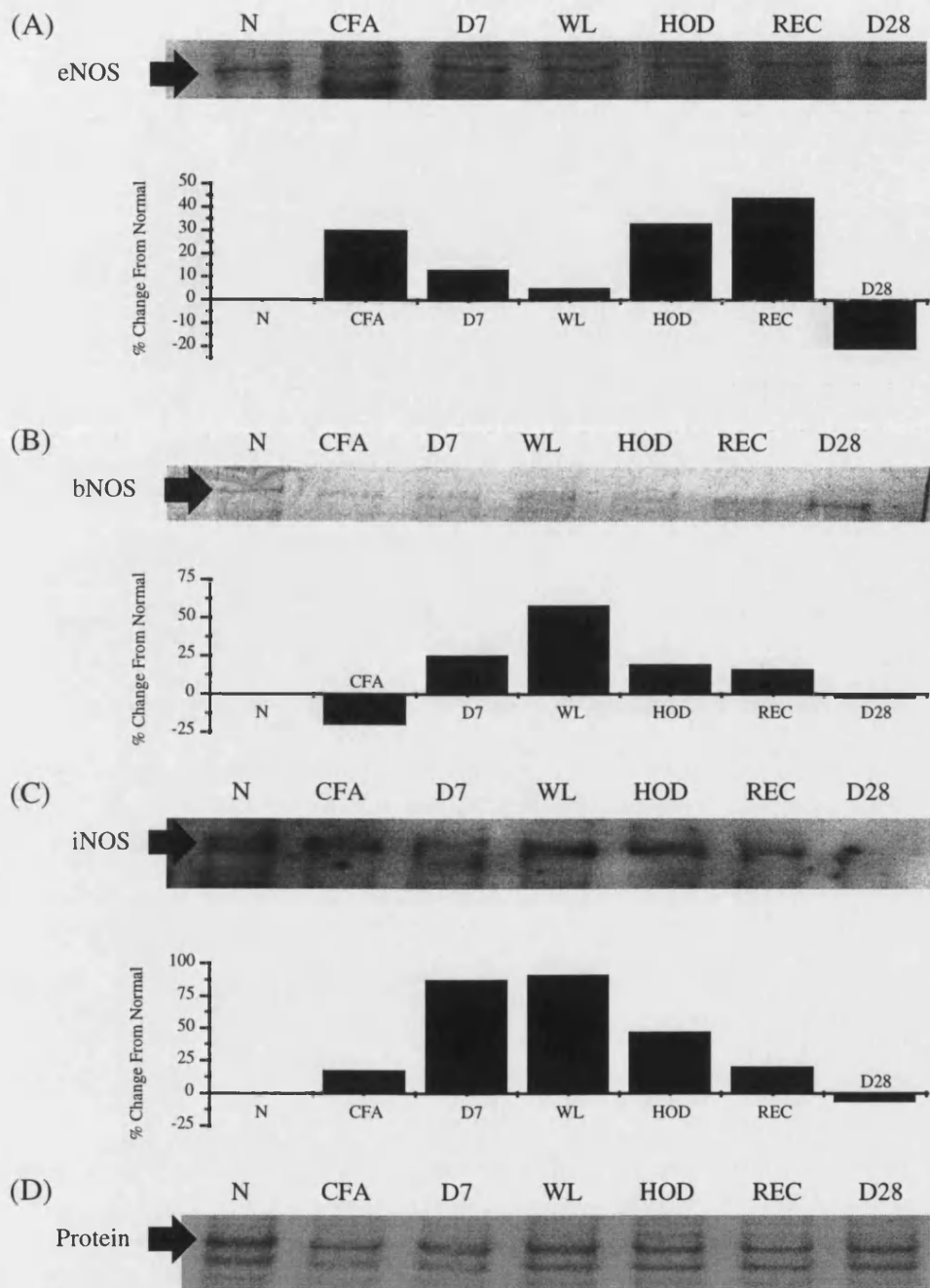


Figure 3.6 EAE disease profile of medulla-pons NOS protein expression

CNS cytosol was obtained and pooled from the medulla-pons of 3 normal Lewis rats (N); CFA-inoculated Lewis rats (CFA) and EAE-inoculated animals at day 7 P.I. (D7); weight loss (WL); height of disease (HOD); recovery (REC) and day 28 P.I. (D28). CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with (A) anti-eNOS; (B) anti-bNOS and (C) anti-iNOS antibodies. NOS protein was detected using ECL (A & C) or PAP complex (B). Densitometric analysis of blots and SDS-gels (D) was undertaken and following correction for differences in total protein content NOS levels were expressed as % change from normal value. Results shown are representative of 3 separate experiments.

inoculated rats expressed low levels of bNOS enzyme in medulla-pons samples (20 % below normal) (Figure 3.6b).

Expression of iNOS protein in medulla-pons tissue from EAE-inoculated rats followed a similar pattern to that of bNOS (Figure 3.6). Levels of iNOS were increased in medulla-pons cytosol preparations throughout active EAE. However, maximal levels were attained before disease signs were displayed (day 7 P.I. 87 %, weight loss 91 % above normal) (Figure 3.6c). Following weight loss the amount of iNOS protein expressed in the medulla-pons of EAE-diseased animals steadily diminished and had fallen to below normal levels by day 28 P.I. (height of disease 47 %, recovery 20 % above normal, day 28 P.I. 7 % below normal) (Figure 3.6c). By comparison, CFA-inoculated Lewis rats expressed increased levels of iNOS in medulla-pons preparations (17 % above normal) (Figure 3.6c).

3.4.3 EAE Disease Profile of Cervical Spinal Cord NOS Protein Expression

Basal levels of NOS enzymes were detected in cervical spinal cord tissues of normal animals (Figure 3.7). In addition, both CFA-inoculated and EAE-sensitised rats contained NOS enzymes in cytosol samples prepared from cervical spinal tissue. However, the amounts of each enzyme varied and no correlation between NOS enzyme expression and EAE severity could be determined (Figure 3.7).

Although eNOS expression was enhanced in the cervical spinal cord during EAE as the disease progressed the levels of eNOS protein present decreased (day 7 P.I. 71 %, weight loss 28 %, height of disease 28 % above normal) (Figure 3.7a). Furthermore, after day 7 P.I. eNOS expression in EAE-diseased rats was not different to that observed in cervical spinal cord cytosol from CFA-inoculated animals (CFA 23 % above normal) (Figure 3.7a). The eNOS protein levels in EAE-sensitised Lewis rats cervical spinal cord continued to diminish during recovery and by day 28 P.I. had fallen below normal levels (recovery 5 % above normal, day 28 P.I. 47 % below normal) (Figure 3.7a).

By comparison, spinal cord samples from EAE-sensitised rats contained lower levels of bNOS than were present in normal animals tissues with the exception of

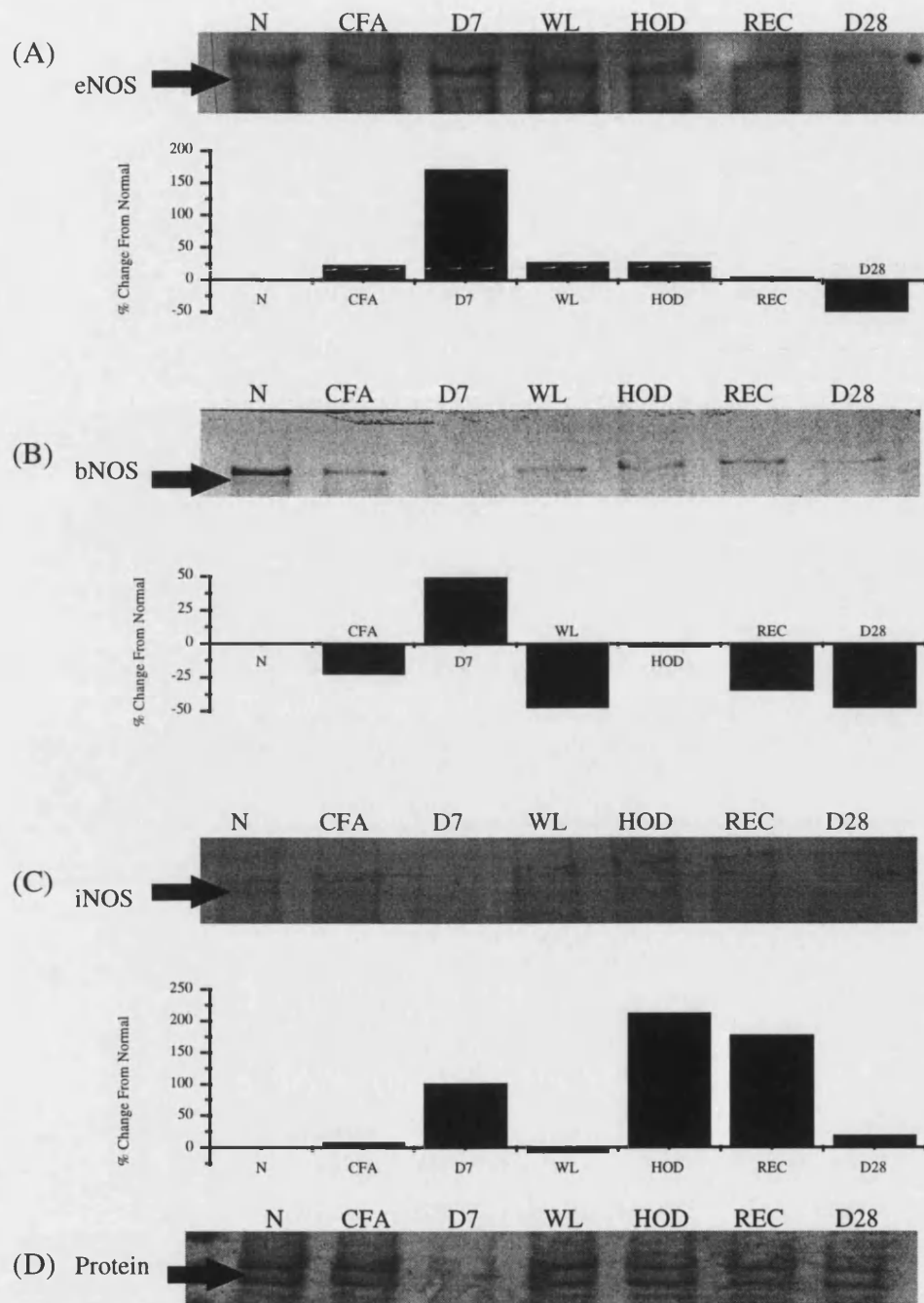


Figure 3.7 EAE disease profile of cervical spinal cord NOS protein expression

CNS cytosol was obtained and pooled from the cervical spinal cord 3 normal Lewis rats (N); CFA-inoculated Lewis rats (CFA) and EAE-inoculated animals at day 7 P.I. (D7); weight loss (WL); height of disease (HOD); recovery (REC) and day 28 P.I. (D28). CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with (A) anti-eNOS; (B) anti-bNOS and (C) anti-iNOS antibodies. NOS protein was detected using PAP complex. Densitometric analysis of blots and SDS-gels (D) was undertaken and following correction for differences in total protein content NOS levels were expressed as % change from normal value. Results shown are representative of 3 separate experiments.

samples obtained 7 days P.I. in which bNOS expression was elevated (49 % above normal) (Figure 3.7b). In addition, CFA-inoculated animals also had lower levels of bNOS enzyme in their cervical spinal cord cytosols than did normal rats (22 % below normal) (Figure 3.7b).

iNOS protein levels in cervical spinal cord cytosol preparations were elevated throughout the course of EAE, with the exception of weight loss at which timepoint iNOS expression was comparable to that of normal animals (weight loss 7 % below normal) (Figure 3.7c). Maximum expression of iNOS was observed when animals displayed peak signs of disease (212 % above normal) (Figure 3.7c). However, iNOS levels remained elevated during recovery with protein expression continuing to be greater than in cervical spinal cord tissues of normal animals at day 28 P.I. (recovery 177 %, day 28 P.I. 19 % above normal) (Figure 3.7c). In contrast, CFA-inoculated rats contained similar amounts of iNOS enzyme in cytosol prepared from spinal tissue as were present in the same tissue of normal animals (8 % above normal) (Figure 3.7c).

3. DISCUSSION

3.1 Nitrite Levels in the CNS During EAE

The precise mechanisms responsible for the CNS damage associated with chronic neurodegenerative diseases such as MS and EAE remain undefined. Nevertheless a clear role for inflammatory mediators released by invading and resident CNS immunocompetent cells has been identified. In particular recent attention has focused on the involvement of NO and ROS in disease pathology.

Studies investigating the role of NO in EAE were initiated by examining the NO content of CNS tissues. However, due to the short half-life of NO the nitrite breakdown product was measured in CNS tissues *ex vivo* as an indicator of NO generation. Nitrite content was measured in the cerebellum, medulla-pons and cervical spinal cord regions as these areas are differentially affected by EAE with respect to BBB permeability and inflammatory cell infiltration (Bolton *et al.* 1984, Paul & Bolton 1995). Indeed during EAE both the medulla-pons and cervical spinal cord develop highly permeable barriers and demonstrate extensive infiltrate accumulation whereas vascular and cellular changes were consistently less extensive in the cerebellum.

Although nitrite levels were detectable in CNS tissues from normal and CFA-control inoculated Lewis rats, neurological EAE was associated with a significant increase in CNS nitrite content. Interestingly nitrite levels have not been determined previously in CNS tissue during EAE. However inflammatory cells isolated from EAE-diseased animals have been shown to generate enhanced levels of reactive nitrogen intermediates (MacMicking *et al.* 1992). Furthermore, increased levels of reactive nitrogen intermediates have been identified in serum samples from EAE-sensitised rats prior to and during symptomatic disease development (Ludowyk *et al.* 1993). The results of the present study demonstrate that nitrite levels in CNS tissue were enhanced during both the induction and active phase of EAE thus suggesting a possible cytotoxic role for the molecule in disease development. In contrast, during the recovery phase CNS extracts from EAE-diseased rats continued to display increased nitrite content which indicates that

NO has a potential role in neuroprotection. Moreover, subsequent to the loss of neurological deficits in EAE CNS nitrite levels returned to normal values hence providing further evidence of a role for NO in neurological disease development and resolution. Indeed the precise involvement of NO in EAE pathogenesis may be better determined by pharmacological manipulation of NO levels in the animal model.

3.2 NO Production in the CNS

Basal levels of nitrite can be readily generated in the CNS of normal animals following the activation of constitutive forms of NOS in endothelial cells, neurons, and astrocytes (Garthwaite 1991, Pollock *et al.* 1992, Murphy *et al.* 1993). Similarly, in the CNS tissue of EAE-diseased animals nitrite will be produced upon cNOS activation. However in addition to basal nitrite synthesis NO can be formed in EAE following the cytokine stimulation of iNOS in resident CNS cells such as microglia, astrocytes and the endothelial cells (Boje & Arora 1992, Galea *et al.* 1992, Simmons & Murphy 1992, Minc-Golomb & Schwartz 1994) together with infiltrating inflammatory cells (Stuehr & Nathan 1989). Nitrite levels varied in each CNS region examined not only in EAE-sensitised rats but also in normal animals. This may be accounted for, in part, by differences in NOS enzyme activity throughout the CNS, or may be due to variations in the amount of NOS enzyme present in each CNS region. Förstermann *et al.* (1990) have previously demonstrated differential activity of NOS in CNS tissues. Moreover, immunohistochemical studies have provided evidence of a distinctive distribution of NOS in the CNS (Bredt & Snyder 1990, Bredt *et al.* 1990). In addition, during EAE inflammatory cell infiltration differs between the cerebellum, medulla-pons and cervical spinal tissues (Bolton *et al.* 1984). Therefore, the relative availability of NOS may vary between these areas due to differences in the number of NO-generating cells present.

3.3 NOS Expression Throughout EAE

In order to elucidate the relative contribution of each NOS isoform in the generation of CNS-derived NO, cytosol fractions from EAE-diseased animals were examined for the presence of e, b and iNOS using Western blotting techniques. While other investigators have demonstrated the expression of iNOS mRNA in CNS tissues derived from EAE-sensitised rats (Koprowski *et al.* 1993, Hooper *et al.* 1995, Okuda *et al.* 1995, Van Dam *et al.* 1995) NOS protein levels have not been assessed in EAE-diseased tissues. The EAE profile of protein expression was not only dependent on the CNS tissues examined but also on the NOS isoform detected. For example, while the iNOS levels detected in the cerebellum tissue of EAE-inoculated animals correlated with neurological disease development, medulla-pons and cervical spinal cord levels of iNOS peaked at the induction and the active/recovery phases of EAE respectively. Furthermore, whereas the cerebellum levels of bNOS determined during EAE corresponded to disease progression, no such association was apparent between EAE and the bNOS levels detected in the medulla-pons and cervical spinal cord regions. These variations may be due to differences in the cellular composition of the areas analysed throughout the disease process. Indeed the presence of NOS enzymes in the CNS tissues of EAE-sensitised rats will not only be dependent upon the availability of NOS in resident CNS cells but will also be determined by infiltrating inflammatory cell NOS formation. Although the levels of e, b and iNOS varied in each CNS region throughout EAE the NOS protein levels were generally greater than normal values during the induction, active and recovery phases of EAE. However, no difference existed between NOS protein levels in the CNS of normal animals and EAE-inoculated Lewis rats sampled 28 days P.I. Moreover, the NOS enzyme expression during EAE corresponded with the nitrite levels measured in CNS tissues of EAE-diseased animals as nitrite levels were also elevated throughout the induction, active and recovery phases but within normal limits by day 28 P.I.

In conclusion, neurological development of EAE was associated with an upregulation of e, b and iNOS enzyme expression. While iNOS expression in the CNS is known to be readily induced under certain conditions (Galea *et al.* 1992) protein levels for

e and bNOS are thought to be constitutively regulated (Brune & Lapetina 1991). However, recent reports have suggested that the transcription of eNOS may also be regulated by a variety of factors, including cytokines and sex hormones (Sessa 1994, Förstermann & Kleinert 1995) which may provide an explanation for the enhanced expression of eNOS observed in the CNS of EAE-sensitised animals. Similarly the expression of bNOS can be altered under certain circumstances such as cellular injury (Verge *et al.* 1992, Regidor *et al.* 1993), which may account for the upregulation of bNOS identified in CNS tissues from EAE-inoculated Lewis rats.

As e, b, and iNOS protein levels were enhanced during EAE it was not possible to determine which isoforms are responsible for disease development. Indeed the expression of NOS protein does not indicate functional activity. Therefore, in order to ascertain the importance of each NOS isoform in the generation of CNS derived NO during EAE further work is required to determine the specific enzyme activity of all 3 isoforms in CNS tissues of EAE-inoculated rats. Furthermore administration of isoform selective NOS inhibitors to EAE-sensitised animals may facilitate the elucidation of the role of individual NOS enzymes in EAE pathogenesis.

Several investigators have proposed a role for iNOS induction in the development of neurological EAE. iNOS mRNA levels correlate with the severity of EAE (Koprowski *et al.* 1993, Okuda *et al.* 1995), and an abundance of iNOS positive cells have been identified in spinal cord tissue of EAE-diseased animals (Okuda *et al.* 1995). In addition, recent studies have established that iNOS expression can be induced by encephalitogenic lymphoid cells and their secreted products (Misko *et al.* 1995, Hewett *et al.* 1996). Further evidence of iNOS involvement in the pathogenesis of EAE is provided by the studies of Cross *et al.* (1994) and Zhao and co-workers (1996) who demonstrated that selective inhibition of iNOS in EAE-sensitised animals prevented neurological disease development. However, a role for bNOS in neurodegenerative disorders has also been implicated by other investigators (Dawson *et al.* 1991).

3.4 Cytotoxic Effects of NO in EAE

Irrespective of which isoform of NOS is activated, the resultant NO functions as a cytotoxic molecule in a similar fashion. Cytotoxic reactions generated by NO may be mediated via several mechanisms. Toxicity of NO has been attributed, at least in part, to the molecule's ability to cause nitrosylation of iron-sulphur protein (Drapier & Hibbs 1988). Iron-sulphur proteins are important for the functioning of enzymes involved in the Krebs's cycle, the mitochondrial respiratory chain and DNA synthesis and repair (Henry *et al.* 1991, Wink *et al.* 1991). Furthermore interference of mitochondrial respiratory chain enzymes by NO has been implicated as an effector mechanism in neurodegeneration, and in particular EAE (Cleeter *et al.* 1994, Zielasek *et al.* 1995b). In addition to direct cytotoxic effects NO may react with superoxide to form the highly toxic peroxynitrite (Beckman *et al.* 1990). Peroxynitrite or its breakdown products are capable of causing lipid peroxidation (Radi *et al.* 1991) as well as mediating a loss of cellular integrity (Lipton *et al.* 1993). NO may also mediate cytotoxicity through activation of poly(ADP-ribose)synthetase (Zhang *et al.* 1994a) which depletes β -nicotinamide adenine dinucleotide and ATP to cause cell death (Cosi *et al.* 1994, Zhang *et al.* 1994a). Alternatively NO may cause neurodegeneration by increasing the susceptibility of cells to TNF- α toxicity via guanylate cyclase activation and cGMP accumulation (Higuchi *et al.* 1990).

In addition to the general cytotoxic effects of NO, CNS damage in EAE may occur through more disease specific mechanisms of neurotoxicity. Indeed NO has been shown to activate cyclooxygenase (Corbett *et al.* 1993a, Salvemini *et al.* 1993), the enzyme responsible for prostaglandin synthesis. Furthermore as prostaglandin formation has been implicated in EAE pathogenesis (Mertin & Stackpoole 1981) NO may mediate indirect effects on EAE development by enhancing prostaglandin production. Moreover it has been established that NO induces mRNA expression for TNF- α (Magrinat *et al.* 1992, Lander *et al.* 1993), a cytotoxic cytokine which has been associated with the aetiology of EAE (Ruddle *et al.* 1990, Selmaj *et al.* 1991). Therefore NO may indirectly influence the neurological disease development. Finally, due to the chemotactic effects of NO on

monocytes, NO may induce damage in EAE via the CNS recruitment of monocytes (Kaplan *et al.* 1989, Belenky *et al.* 1993).

Clearly the cytotoxic potential of NO production in neurodegenerative disorders is vast. Extensive *in vitro* evidence exists to implicate NO as a primary mediator of neuronal cell death (Boje & Arora 1992, Dawson *et al.* 1993, Skaper *et al.* 1995, Palluy & Rigaud 1996). Additionally, NO has also been proposed as an effector molecule in microglial (Dawson *et al.* 1994, Mitrovic *et al.* 1994a,b, Skaper *et al.* 1996) and oligodendrocyte (Merrill *et al.* 1993, Mackenzie-Graham *et al.* 1994, Mitrovic *et al.* 1995) cytotoxicity.

Although substantial *ex vivo* evidence indicates a cytotoxic role for NO in EAE pathogenesis (MacMicking *et al.* 1992, Koprowski *et al.* 1993, Lin *et al.* 1993, Ludowyk *et al.* 1993, Hooper *et al.* 1995, Misko *et al.* 1995, Okuda *et al.* 1995, Van Dam *et al.* 1995, Hewett *et al.* 1996), *in vivo* studies involving the pharmacological manipulation of NO in EAE have produced conflicting results (Cross *et al.* 1994, Zielasek *et al.* 1995a, Ruuls *et al.* 1996, Zhao *et al.* 1996). The remainder of this investigation attempts to clarify the precise role of NO in the neurological development of actively induced EAE.

CHAPTER 4

QUANTITATION OF ROS

IN EAE

4. RESULTS

Previous studies have demonstrated that, in the absence of L-arginine, NOS can generate ROS such as superoxide and hydrogen peroxide (Meyer *et al.* 1991, Heinzl *et al.* 1992). The current study has shown that NOS enzyme expression was enhanced in the CNS of EAE-sensitised animals. Therefore, disease pathogenesis may be mediated by the formation of ROS by this enzyme. Although pharmacological evidence exists to implicate ROS in EAE pathogenesis (Bowern *et al.* 1984; Honegger *et al.* 1989b; Ruuls *et al.* 1995) *ex vivo* measurements of superoxide and hydrogen peroxide in CNS tissue from diseased animals have not been undertaken. Therefore, superoxide and hydrogen peroxide levels were determined in CNS cytosol fractions from EAE-inoculated Lewis rats to investigate a role for ROS in the aetiology of the disease.

4.1 Superoxide Levels in CNS Cytosol

Superoxide levels were measured in the cerebellum, medulla-pons and cervical spinal cord regions. Levels of superoxide in the cervical spinal cord from normal Lewis rats were in excess of values recorded in the cerebellum and medulla-pons (cerebellum 7 ± 2 , medulla-pons 9 ± 2 , cervical spinal cord 14 ± 3 n moles superoxide/mg protein \pm S.E.M., n=7) (Figure 4.1). Superoxide content in CNS tissues from CFA-inoculated rats were comparable to values recorded in normal animals (cerebellum 9 ± 2 , medulla-pons 11 ± 3 , cervical spinal cord 17 ± 4 , n=10) (Figure 4.1). However, established neurological EAE was associated with a significant increase in superoxide levels in all isolated CNS preparations compared to basal values (cerebellum 55 ± 15 , $p < 0.001$; medulla-pons 50 ± 11 , $p < 0.01$; cervical spinal cord 45 ± 10 , $p < 0.05$, n=10) (Figure 4.1). In addition, superoxide levels in CNS tissue from EAE-diseased animals were significantly elevated compared to values obtained in CFA-inoculated rats (cerebellum $p < 0.01$, medulla-pons $p < 0.05$, cervical spinal cord $p < 0.05$).

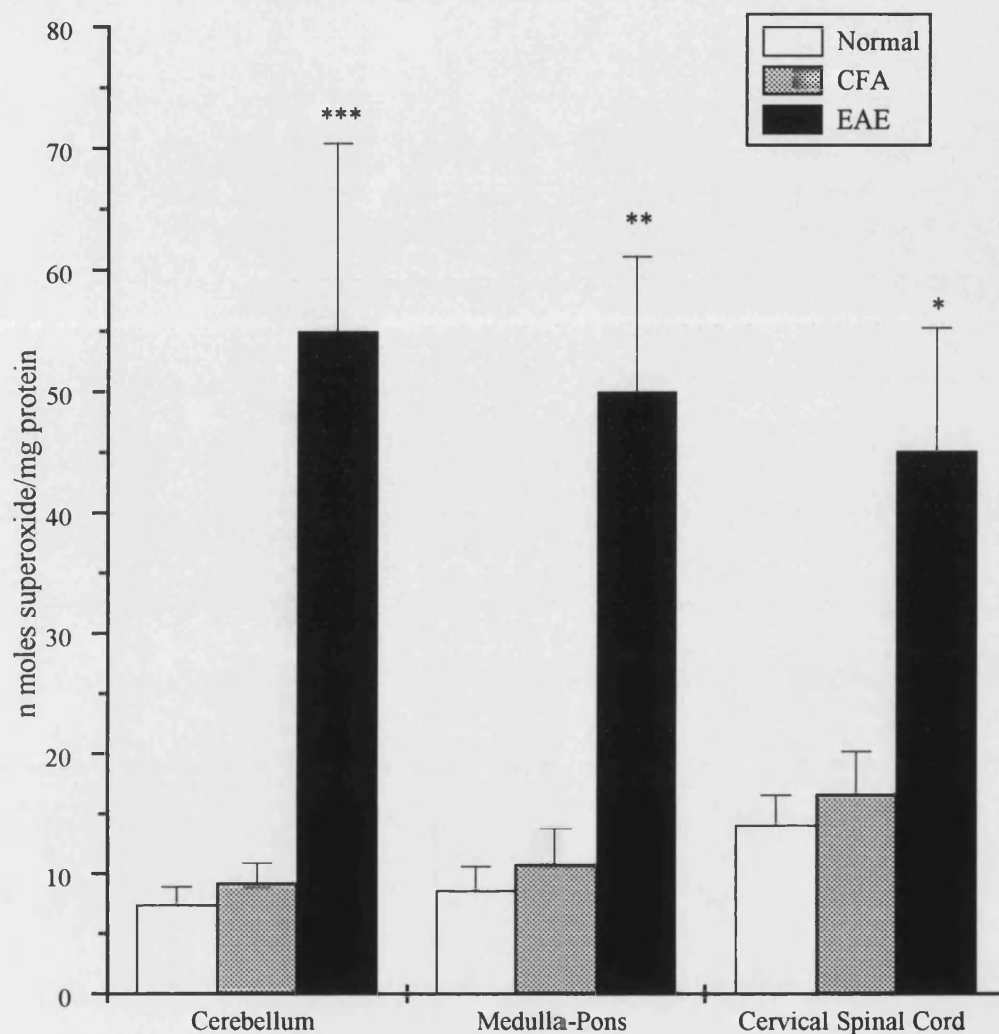


Figure 4.1 Superoxide levels in CNS cytosol.

CNS cytosol samples were prepared from the cerebellum, medulla-pons and cervical spinal cord regions of each animal. Superoxide levels were determined in each sample by measuring the reduction of ferricytochrome C and expressed as n moles superoxide/mg protein \pm S.E.M. Normal Lewis rats, n = 7; CFA control inoculated Lewis rats, sampled day 13 P.I., n = 6; EAE-sensitised Lewis rats, sampled day 13 P.I., n = 10. *p<0.05, **p<0.01, ***p<0.001 Mann Whitney U test compared to normal animals.

4.2 EAE Profile of Superoxide Levels in CNS Cytosol

Superoxide levels were determined in CNS cytosol from EAE-sensitised animals sacrificed at various time-points throughout the disease process in order to establish a correlation between CNS superoxide levels and disease development.

Interestingly a similar profile of superoxide content was obtained in the areas of the CNS studied (Figure 4.2). Levels of superoxide in cerebellum cytosol preparations from EAE-sensitised animals sampled 7 days P.I. were not significantly different to normal values (day 7 P.I. 12 ± 2 vs normal 7 ± 2 , $n=8$ and 7 respectively) (Figure 4.2a). In contrast, cerebellar cytosol fractions from rats sacrificed at weight loss or peak disease severity had significantly elevated superoxide levels (weight loss 69 ± 9 , $p<0.001$, $n=7$; height of disease 55 ± 15 , $p<0.001$, $n=10$) with maximum amounts recorded prior to the onset of disease symptoms (Figure 4.2a). Furthermore, cerebellar cytosol samples from recovered EAE animals also contained increased levels of superoxide (29 ± 8 , $n=7$). However, superoxide content of cerebellar tissue had returned to normal by 28 days P.I. (14 ± 2 , $n=10$) (Figure 4.2a).

Medulla-pons cytosol from EAE-inoculated animals sacrificed at day 7 P.I. contained elevated amounts of superoxide (day 7 P.I. 31 ± 9 , $n=8$; normal 9 ± 2 , $n=7$) (Figure 4.2b). In addition, significantly increased superoxide levels were observed in medulla-pons tissue from sensitised Lewis rats prior to and during the development of symptoms (weight loss 62 ± 8 , $p<0.01$, $n=7$; height of disease 50 ± 11 , $p<0.01$, $n=10$). Maximal superoxide content was recorded before the expression of neurological deficits (Figure 4.2b). During recovery from EAE, cytosol levels of superoxide in the medulla-pons were not significantly elevated above normal values (17 ± 3 , $n=7$). Moreover, superoxide content of medulla-pons tissue at day 28 P.I. was comparable to levels recorded during recovery (13 ± 3 , $n=10$) (Figure 4.2b).

In cervical spinal cord preparations, an almost identical profile of superoxide content and disease severity to that obtained in the cerebellum was identified (Figure 4.2). Superoxide levels in cervical spinal tissues from EAE-sensitised animals sacrificed at day 7 P.I. were within normal limits (day 7 P.I. 15 ± 4 , $n=8$; normal 14 ± 3 , $n=7$).

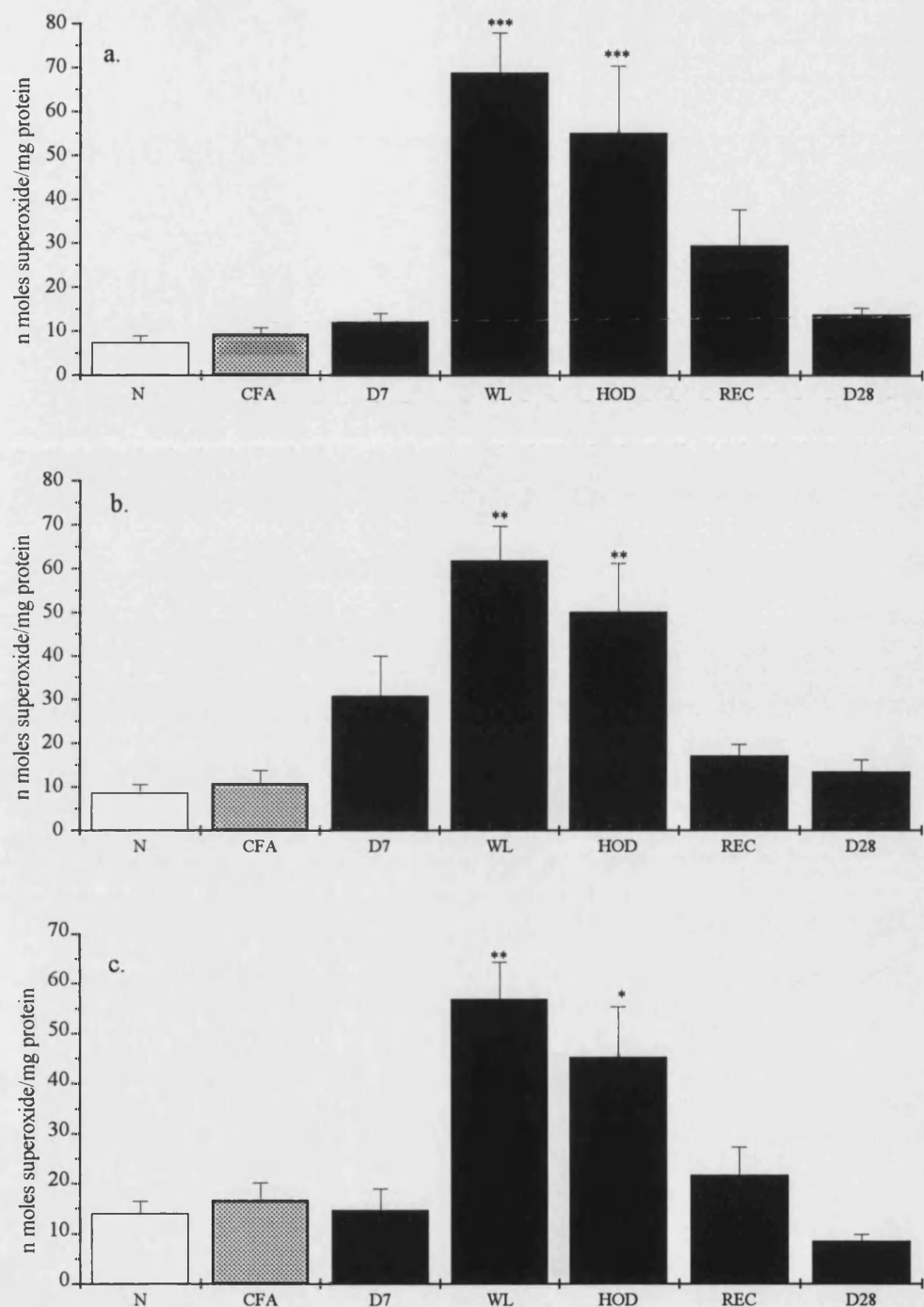


Figure 4.2 EAE profile of superoxide levels in CNS cytosol.

CNS cytosol was prepared from the a. cerebellum, b. medulla-pons and c. cervical spinal tissues from each animal. Superoxide levels were measured by ferricytochrome C reduction and expressed as n moles superoxide/mg protein \pm S.E.M. Normal Lewis rats (N), n = 7; CFA inoculated rats, sampled day 13 P.I., n = 6 and EAE-sensitized Lewis rats sampled at day 7 P.I. (D7), n = 8; weight loss (WL), n = 7; height of disease (HOD), n = 10; recovery (REC), n = 7 and day 28 P.I. (D28), n = 10. *p<0.05, **p<0.01, ***p<0.001 Mann Whitney U test compared to normal animals.

However, during weight loss and peak disease superoxide content of cervical spinal cord cytosol was significantly elevated (weight loss 57 ± 8 , $p < 0.01$, $n=7$; height of disease 45 ± 10 , $p < 0.05$, $n=10$) (Figure 4.2c). The amount of superoxide in the cervical spinal cord had decreased to normal limits during the recovery phase of EAE (22 ± 6 , $n=7$) and by day 28 P.I. were below values recorded in normal animals (8 ± 1 , $n=10$) (Figure 4.2c).

4.3 Hydrogen Peroxide Levels in CNS Cytosol

NO has been shown to exert a cytoprotective effect in CNS inflammation by scavenging superoxide radicals (Wink *et al.* 1994a). NO and superoxide interact to form peroxynitrite molecules which decompose to nitrogen dioxide and hydroxyl radicals. As the reaction of superoxide and NO proceeds faster than the dismutation of superoxide by SOD, hydrogen peroxide levels decrease in the presence of excess NO (Wink *et al.* 1994a). Therefore the hydrogen peroxide content of CNS cytosol fractions was determined to investigate whether the increased amounts of NO observed during EAE scavenges superoxide which would be indicated by a decrease in hydrogen peroxide levels.

Hydrogen peroxide content was assessed in the cerebellum, medulla-pons and cervical spinal tissues. Levels of hydrogen peroxide were similar in all CNS areas isolated from normal animals (cerebellum 2 ± 0.6 , medulla-pons 2 ± 0.5 , cervical spinal cord 2 ± 0.5 μ moles hydrogen peroxide/mg protein \pm S.E.M., $n=10$) (Figure 4.3). Furthermore, hydrogen peroxide content of CNS tissues from CFA-inoculated rats were not significantly different to values recorded in normal animals (cerebellum 4 ± 2 , medulla-pons 6 ± 2 , cervical spinal cord 7 ± 3 , $n=7$) (Figure 4.3). Neurological EAE was associated with a significant increase in CNS cytosol hydrogen peroxide levels (cerebellum 10 ± 2 , $p < 0.05$; medulla-pons 9 ± 2 , $p < 0.01$; cervical spinal cord 5 ± 1 , $p < 0.05$, $n=6$) (Figure 4.3). However the values recorded were similar to those obtained from CFA-inoculated rats.

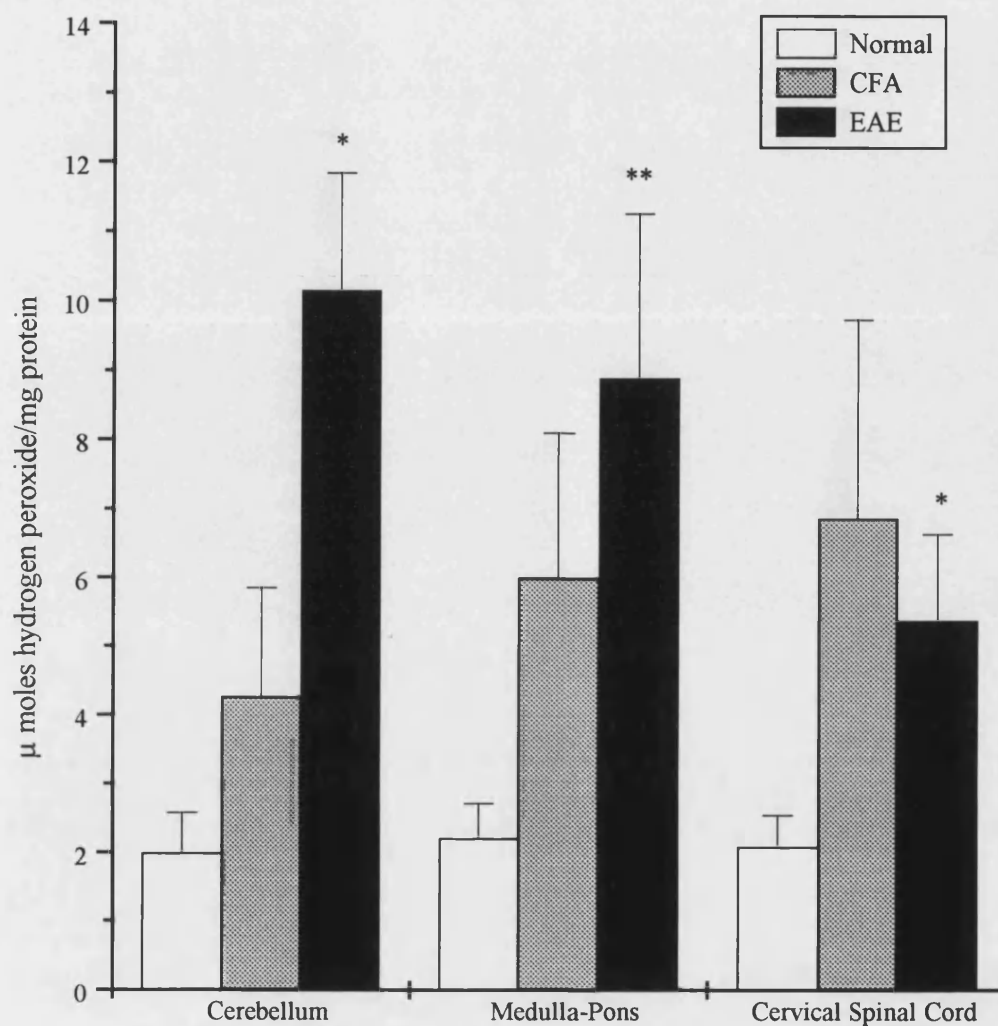


Figure 4.3 Hydrogen peroxide levels in CNS cytosol.

CNS cytosol samples were prepared from the cerebellum, medulla-pons and cervical spinal cord regions of each animal. Hydrogen peroxide levels were measured in each sample using the oxidation of scopoletin and results were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M. Normal Lewis rats, $n = 10$; CFA control inoculated Lewis rats, sampled on day 13 P.I., $n = 7$ and EAE-diseased Lewis rats, sampled on day 13 P.I., $n = 6$. * $p < 0.05$, ** $p < 0.01$ Mann Whitney U test compared to normal animals.

4.4 EAE Profile of Hydrogen Peroxide Levels in CNS Cytosol

Hydrogen peroxide content was determined in CNS cytosol fractions isolated from EAE-inoculated Lewis rats throughout the course of disease to determine a relationship between hydrogen peroxide content and the occurrence of symptoms.

A similar profile of hydrogen peroxide levels and disease development was demonstrated in all CNS areas (Figure 4.4). Hydrogen peroxide content of CNS cytosol samples from EAE-sensitised animals before disease onset were no different to normal values (Figure 4.4). By comparison, the amount of hydrogen peroxide in CNS fractions was significantly elevated when animals displayed peak disease signs (cerebellum 10 ± 2 , $p < 0.05$; medulla-pons 9 ± 2 , $p < 0.01$; cervical spinal cord 5 ± 1 , $p < 0.05$, $n=6$). During recovery the hydrogen peroxide content of CNS tissue was below normal values (cerebellum 1 ± 0.2 , medulla-pons 1 ± 0.1 , cervical spinal cord 1 ± 0.1) but had returned to basal levels by day 28 P.I. (cerebellum 2 ± 0.5 , medulla-pons 2 ± 0.5 , cervical spinal cord 2 ± 0.3 , $n=10$) (Figure 4.4).

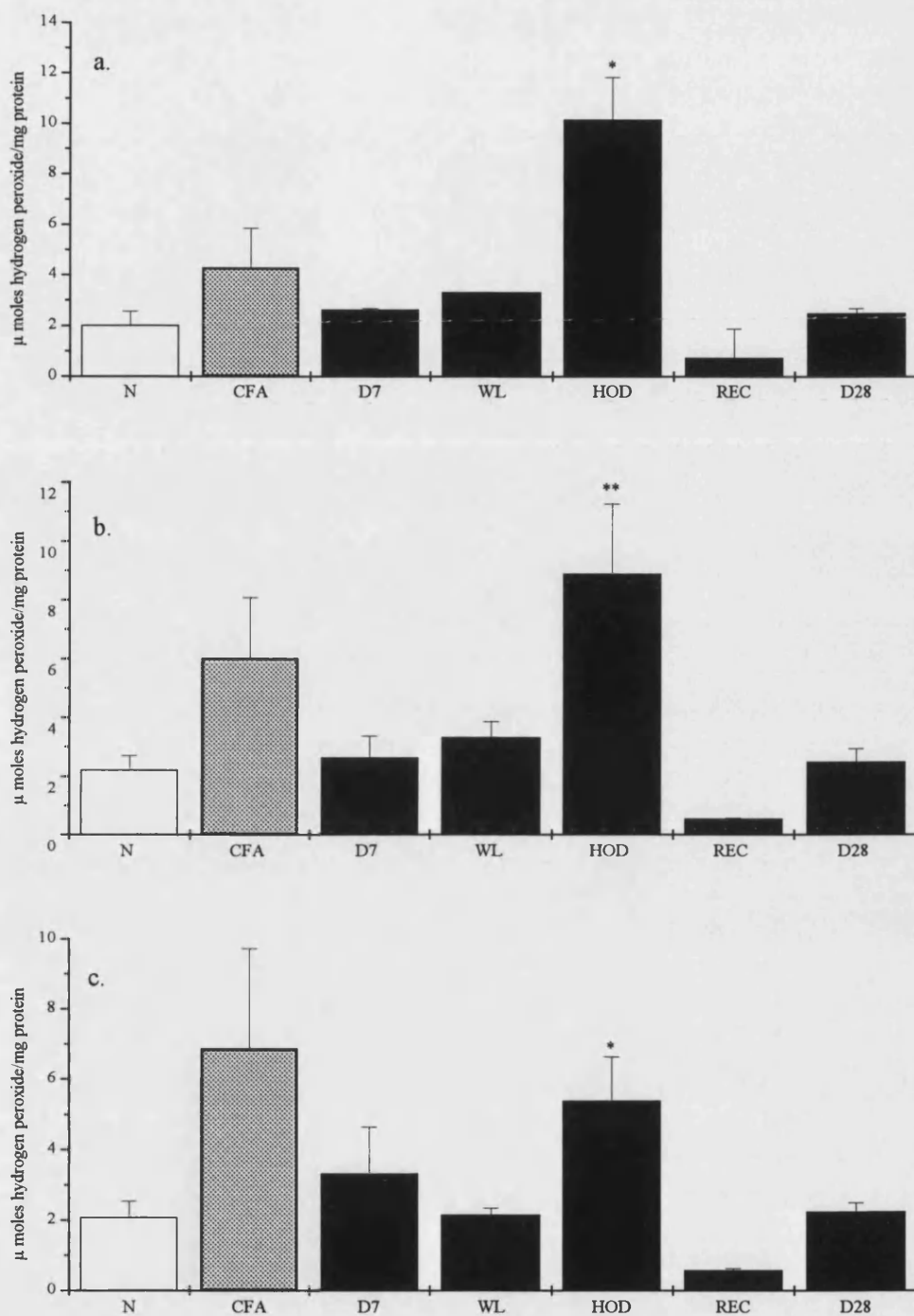


Figure 4.4 EAE profile of hydrogen peroxide levels in CNS cytosol.

CNS cytosol was prepared from the a. cerebellum, b. medulla-pons and c. cervical spinal cord areas of each animals. Hydrogen peroxide levels were measured by oxidation of scopoletin and expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M. Normal Lewis rats (N), n = 10; CFA inoculated rats, sampled day 13 P.I., n = 7 and EAE-sensitized Lewis rats sampled at day 7 P.I. (D7), n = 8; weight loss (WL), n = 7; height of disease (HOD), n = 6; recovery (REC), n = 7 and day 28 P.I. (D28), n = 10. * $p < 0.05$, ** $p < 0.01$ Mann Whitney U test compared to normal animals.

4 DISCUSSION

4.1 ROS in EAE Pathogenesis

Previous work has implicated ROS in the pathogenesis of EAE and MS (Hartung *et al.* 1992, Levine 1992). Indeed much pharmacological evidence exists to demonstrate that scavenging ROS (Lehmann *et al.* 1994, Hansen *et al.* 1995), enhancing ROS degradation (Ruuls *et al.* 1995), or interfering with the effector mechanisms of ROS production (Bowern *et al.* 1984, Karlik *et al.* 1991) prevents disease development in experimental models of EAE. Additional studies have identified enhanced production of ROS from cells isolated from the CNS of EAE-diseased animals (MacMicking *et al.* 1992, Ruuls *et al.* 1995). However, the relative contributions of superoxide and hydrogen peroxide in the overall CNS generation of ROS by EAE-sensitised rats has not been investigated. Thus a protocol was devised to measure CNS levels of superoxide and hydrogen peroxide individually.

Superoxide and hydrogen peroxide levels were determined in the cerebellum, medulla-pons and cervical spinal tissue from normal, CFA-control and EAE-inoculated Lewis rats. Detectable levels of superoxide and hydrogen peroxide were present in the CNS of normal animals, but the development of EAE was accompanied by a significant elevation in ROS content. These results are in agreement with the findings of MacMicking *et al.* (1992) and Ruuls *et al.* (1995) who demonstrated a significant increase in ROS generation by inflammatory cells isolated from the CNS of EAE-diseased animals. In contrast in the current study no significant difference existed between hydrogen peroxide levels in CNS tissue from CFA-control inoculated or EAE-sensitised Lewis rats. The results suggest that the elevation in hydrogen peroxide detected during EAE are not specifically related to disease. By comparison, Ruuls *et al.* (1995) demonstrated, through pharmacological manipulation, that hydrogen peroxide, and not superoxide, was the principal mediator in EAE.

Further evidence to implicate superoxide and not hydrogen peroxide in the pathogenic mechanisms of EAE is provided by the observation that while CNS superoxide

content was maximal prior to disease onset, peak levels of hydrogen peroxide were detected at the height of neurological deficits. As superoxide decays to hydrogen peroxide either spontaneously or via a SOD-catalysed reaction it may be expected that during EAE the levels of both species would correspond. However the superoxide and hydrogen peroxide values recorded did not correlate suggesting an interference in the superoxide dismutation reaction, which may be partly explained by the interaction of NO with superoxide to generate peroxynitrite in a process known to limit the production of hydrogen peroxide (Guitierrez *et al.* 1996). Interestingly unlike NO levels, ROS content of CNS tissue is only elevated during the induction and active phase of EAE with values returning to normal during recovery which provides further evidence of a role for ROS in the neurodestructive processes of EAE.

4.2 ROS Production in the CNS

Several mechanisms of ROS production have been identified in biological tissue including the xanthine oxidase- (Granger *et al.* 1981) and NADPH oxidase- (Tauber *et al.* 1979) catalysed formation of ROS. In addition, auto-oxidation of catecholamines and dopamine results in the synthesis of ROS (Singal *et al.* 1980). ROS can also be produced following NMDA receptor activation (Lafon-Cazal *et al.* 1993a,b). The reasons for an abundance of ROS in the CNS are that metals, which are required for the catalytic formation of free radicals, are present in high concentrations in the brain. Additionally, the CNS is rich in catecholamines and dopamine which can interact with oxygen to produce ROS (Lehmann *et al.* 1994). Furthermore antioxidant mechanisms are moderated in the CNS which would lead to the maintenance of ROS.

4.3 Cytotoxic Effects of ROS

During EAE a variety of resident CNS cells including microglia, astrocytes and neurons (Colton & Gilbert 1987, Chan *et al.* 1988, Dugan *et al.* 1995) together with infiltrating macrophages and other leukocytes (Nathan *et al.* 1983, Nathan 1987) are capable of generating ROS. The resultant ROS formation may mediate neurotoxicity through several mechanisms. One of the primary mechanisms by which ROS are thought to mediate CNS damage is through the initiation of a chain reaction of lipid peroxidation (Radi *et al.* 1991). Indeed myelin, present in a lipid rich environment, is particularly vulnerable to peroxidation due to the abundance of iron, which catalyses free radical reactions (Halliwell *et al.* 1989). Moreover it has been reported that lipid peroxidation is activated during EAE (Stepanenko *et al.* 1984).

CNS-derived ROS may also contribute to the pathogenesis of neurodegenerative disorders through cytotoxic actions on oligodendrocytes (Groit *et al.* 1990, Kim & Kim 1991). Although the CNS production of ROS may lead to demyelination minimal myelin breakdown is observed in the acute EAE model utilised in the present study. Therefore the results strongly suggest that ROS may exert other effects in the CNS to mediate neurological disease development.

Breakdown of the BBB is a well recognised event in the pathogenesis of EAE (Liebowitz 1969). However, the precise mechanisms involved in loss of neurovascular integrity have not been fully elucidated. ROS have been proposed as mediators of cerebroendothelial disruption in non-immune models of BBB breakdown (Shukla *et al.* 1993). Hence the ROS generated in the CNS during EAE may contribute to disease development through deleterious effects on neurovascular permeability. Similarly the ROS mediated vascular disruption and endothelial cell damage results in oedema formation (Butler *et al.* 1985), which has been closely associated with the aetiology of EAE (Wajda 1972).

ROS may also mediate CNS damage in EAE via peroxynitrite formation. Peroxynitrite is a powerful oxidant which is generated upon the reaction of superoxide with NO (Beckman *et al.* 1990). As endothelial cells (Beckman *et al.* 1990), brain cells

(Pou *et al.* 1992) and inflammatory cells (Nathan 1987, Rubanyi *et al.* 1991) are capable of producing both NO and superoxide the potential for peroxynitrite formation is substantial. The generation of hydroxyl-radical-like oxidants is only one of several cytotoxic reactions carried out by peroxynitrite (Beckman *et al.* 1990) which may initiate peroxidation (Radi *et al.* 1991), inactivate sodium channels (Bauer *et al.* 1992) or react with transition metals to form a powerful nitrating agent (Ischiropoulos *et al.* 1992). In part, peroxynitrite production may induce EAE through effects on the BBB. Endothelial cell damage occurs following exposure to peroxynitrite (Beckman *et al.* 1990, Volk *et al.* 1996) which may contribute to the neurovascular disruption and subsequent events involved in EAE pathogenesis.

Although the interaction of NO and superoxide is thought to result in the generation of a molecule with a greater cytotoxic potential than either species alone some studies contradict this proposal. Indeed several investigations have demonstrated that the reaction between superoxide and NO may exert a protective effect in pathological disorders (Kubes *et al.* 1991, Choi 1993, Wink *et al.* 1993a). Therefore as both molecules are produced in excess in EAE their precise involvement in disease pathogenesis cannot be confirmed in light of recent observations.

CHAPTER 5

IMMUNOSUPPRESSIVE

AGENTS

5. RESULTS

Immunosuppressive agent such as Dex and CsA have previously been shown to inhibit EAE-development (Komark & Dietrich 1971, Bolton *et al.* 1982a,b). However, the precise mechanisms through which these drugs exert their effects have not been fully characterised. Therefore, the effects of Dex and CsA administration on CNS nitrite, superoxide and hydrogen peroxide levels in EAE-inoculated Lewis rats were determined.

5.1 Effect of Immunosuppressive Agents on Body Weight Loss and Neurological Development of EAE

EAE-sensitised Lewis rats were either injected s.c. with Dex (1 mg/kg body weight) twice daily or orally dosed with CsA (50 mg/kg body weight) once daily for two days from disease associated weight loss. The doses of Dex and CsA utilised have been demonstrated as effectively inhibiting EAE (Paul & Bolton 1995). Additionally, EAE-inoculated animals were treated with the appropriate vehicle. Dosed and undosed animals were observed daily for body weight changes (Figure 5.1) and neurological disease signs (Table 5.1).

Administration of Dex to EAE-diseased rats significantly reduced disease severity ($p<0.01$) and the development of paralytic symptoms ($p<0.05$) without affecting the incidence or onset of neurological EAE (Table 5.1). However, treatment of EAE-inoculated animals with PBS vehicle had no effect on the onset or development of neurological disease (Table 5.1). In addition, the profile of body weight changes in dexamethasone and vehicle treated rats were similar to fluctuations recorded in undosed sensitised animals (Figure 5.1a).

Treatment with CsA delayed the onset of EAE ($p<0.01$), alleviated disease severity ($p<0.001$) and dramatically reduced paralytic symptoms ($p<0.01$) (Table 5.1). By comparison, neurological disease parameters were unaffected by the administration of

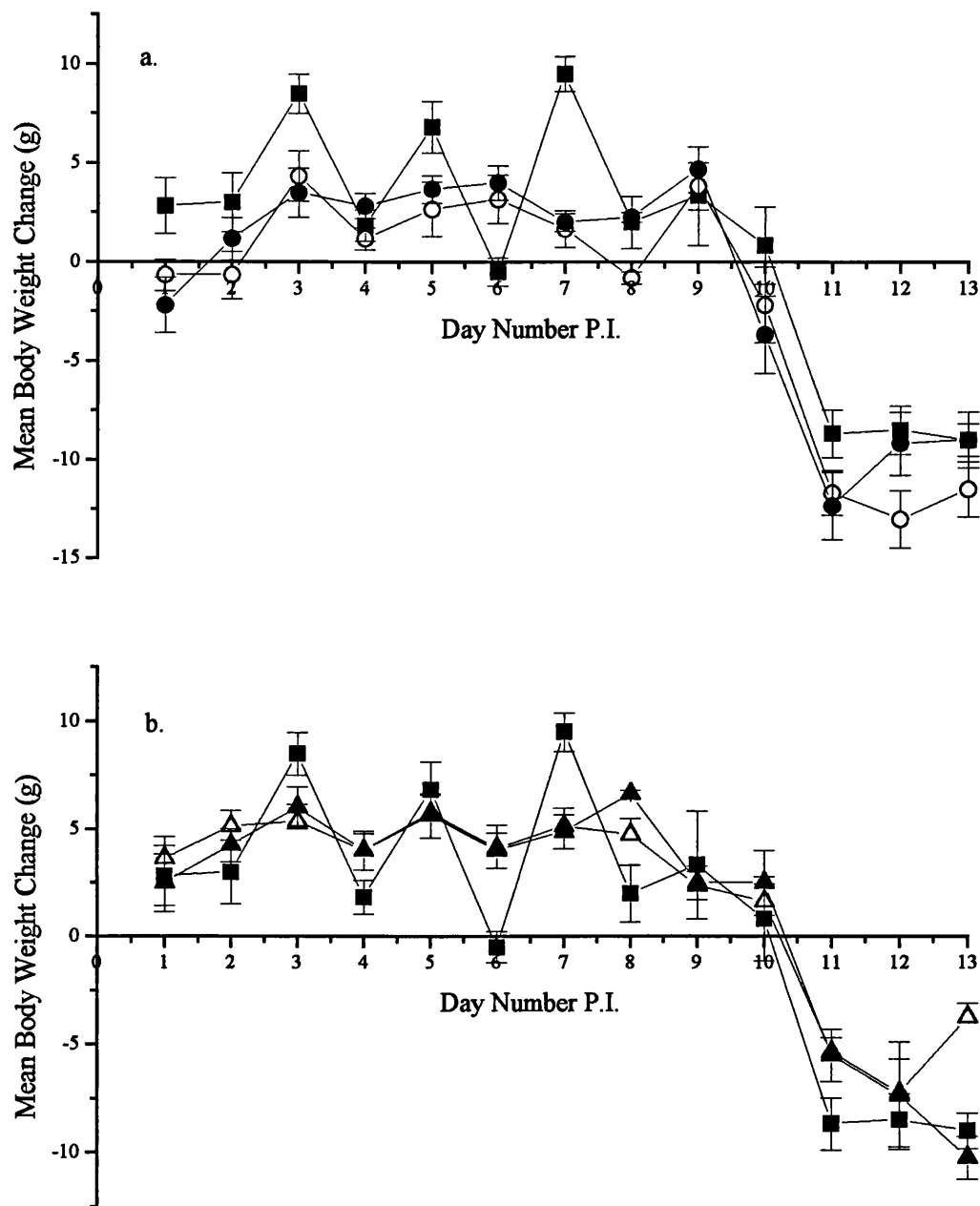


Figure 5.1 Effect of Dex and CsA administration on body weight changes

Animals were weighed daily. Body weight changes were measured in g and results expressed as mean body weight changes \pm S.E.M.

a. (—■—) EAE-diseased Lewis rats, n=6; (—○—) EAE-inoculated animals s.c. injected with Dex (1mg/kg body weight) twice daily for 2 days from disease associated weight loss, n=6; (—●—) EAE-sensitised rats injected s.c. with vehicle (PBS, 1ml/kg body weight) twice daily for 2 days commencing at disease associated weight loss, n=6.

b. (—■—) EAE-diseased Lewis rats, n=6; (—△—) EAE-sensitised animals orally dosed with CsA (50 mg/kg body weight) once daily for 2 days from disease associated weight loss, n=9; (—▲—) EAE-inoculated rats orally dosed with vehicle (extra virgin olive oil, 2 ml/kg body weight) once daily for 2 days commencing on disease associated weight loss, n=10.

Table 5.1 Neurological symptoms in EAE-sensitised Lewis rats following treatment with Dex (1 mg/kg body weight, s.c., twice daily for 2 days from disease associated weight loss), Dex vehicle (PBS, 1 ml/kg body weight, s.c. twice daily for 2 days from disease associated weight loss), CsA (50 mg/kg body weight, orally for 2 days from disease associated weight loss) and CsA vehicle (extra virgin olive oil, 2 ml/kg body weight, orally for 2 days from disease associated weight loss).

Group	Incidence Of EAE	Mean Day Of Disease Onset \pm S.E.M.	Mean Peak Disease Severity \pm S.E.M.	Number Of Rats Paralysed/Total
EAE	9/9	11 ± 0.2	3 ± 0.3	6/9
Dex	6/6	12 ± 0.3	$1 \pm 0.2^{***\ddagger}$	0/6 ^{***}
Vehicle	6/6	11 ± 0.2	3 ± 0.4	4/6
CsA	8/9	$13 \pm 0.2^{***\ddagger}$	$1 \pm 0.2^{***\ddagger}$	0/9 ^{**}
Vehicle	10/10	12 ± 0.1	2 ± 0.3	3/10

^{**}p<0.01, ^{***}p<0.001 compared to EAE group.

[†]p<0.05, [‡]p<0.01 compared to vehicle group.

Differences in mean day of disease onset and mean peak disease severity were analysed using the Mann Whitney U test.

Differences in the incidence of EAE and the number of rats displaying paralysis were analysed using the Chi-squared test.

vehicle (Table 5.1). Furthermore, the body weight loss experienced by CsA or vehicle treated rats was similar to changes observed in undosed sensitised animals (Figure 5.1b).

5.2 Effect of Immunosuppressive Agents on Nitrite Levels in CNS Cytosol

A possible mechanisms through which immunosuppressive agents exert protective effects in EAE could be via the inhibition of NO formation. Therefore, nitrite levels were determined in CNS cytosol samples from untreated EAE-sensitised animals and rats dosed with dexamethasone, cyclosporin A or the appropriate vehicle.

Dex administration significantly reduced nitrite levels in the cerebellum ($p < 0.01$) and medulla-pons ($p < 0.001$) of EAE-inoculated Lewis rats to within normal limits (Figure 5.2). However, no decrease in nitrite production in cervical spinal cord cytosol was observed following treatment with Dex (Figure 5.2). Moreover, s.c. vehicle treatment of EAE-sensitised animals did not significantly alter CNS cytosol nitrite levels in the preparations assayed (Figure 5.2).

Nitrite levels in CNS cytosol preparations from EAE-diseased rats were significantly diminished by CsA administration (cerebellum $p < 0.001$, medulla-pons $p < 0.001$, cervical spinal cord $p < 0.05$) (Figure 5.3). In contrast, vehicle treatment of EAE-inoculated animals did not affect the nitrite content of the CNS tissues analysed (Figure 5.3).

5.3 Effect of Immunosuppressive Agents on iNOS Protein Expression in CNS Cytosol

Dex and CsA are thought to prevent the formation of iNOS and thus inhibit NO and nitrite production (Rees *et al.* 1990a, Conde *et al.* 1995). Therefore, the ability of these agents to inhibit the production of iNOS protein in the CNS of EAE-diseased animals was assessed. iNOS protein expression was determined in CNS cytosol samples from EAE-sensitised rats treated with Dex, CsA or appropriate vehicle.

Cytosol samples obtained from the cerebellum, medulla-pons and cervical spinal tissues were analysed for the presence of iNOS using SDS-PAGE and immunoblotting

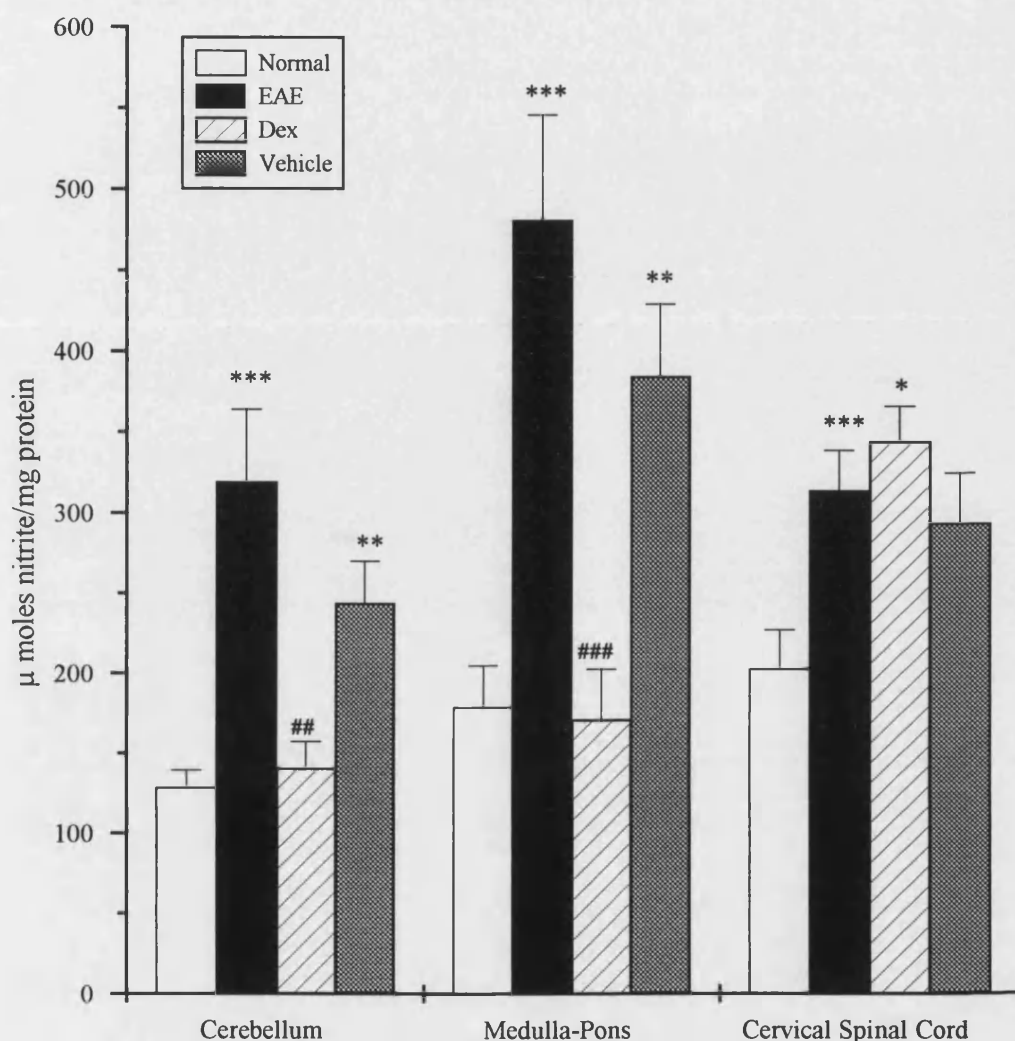


Figure 5.2 Effect of Dex administration on nitrite levels in CNS cytosol
 CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, $n = 12$; EAE-diseased Lewis rats, $n = 12$; EAE-sensitised Lewis rats s.c. dosed with Dex (1mg/kg body weight) twice daily for 2 days from disease associated weight loss, $n = 9$; EAE-inoculated Lewis rats s.c. injected with vehicle (PBS, 1ml/kg body weight) twice daily for 2 days commencing from disease associated weight loss, $n = 7$. ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. ## $p < 0.01$, ### $p < 0.001$ Mann Whitney U test compared to EAE animals.

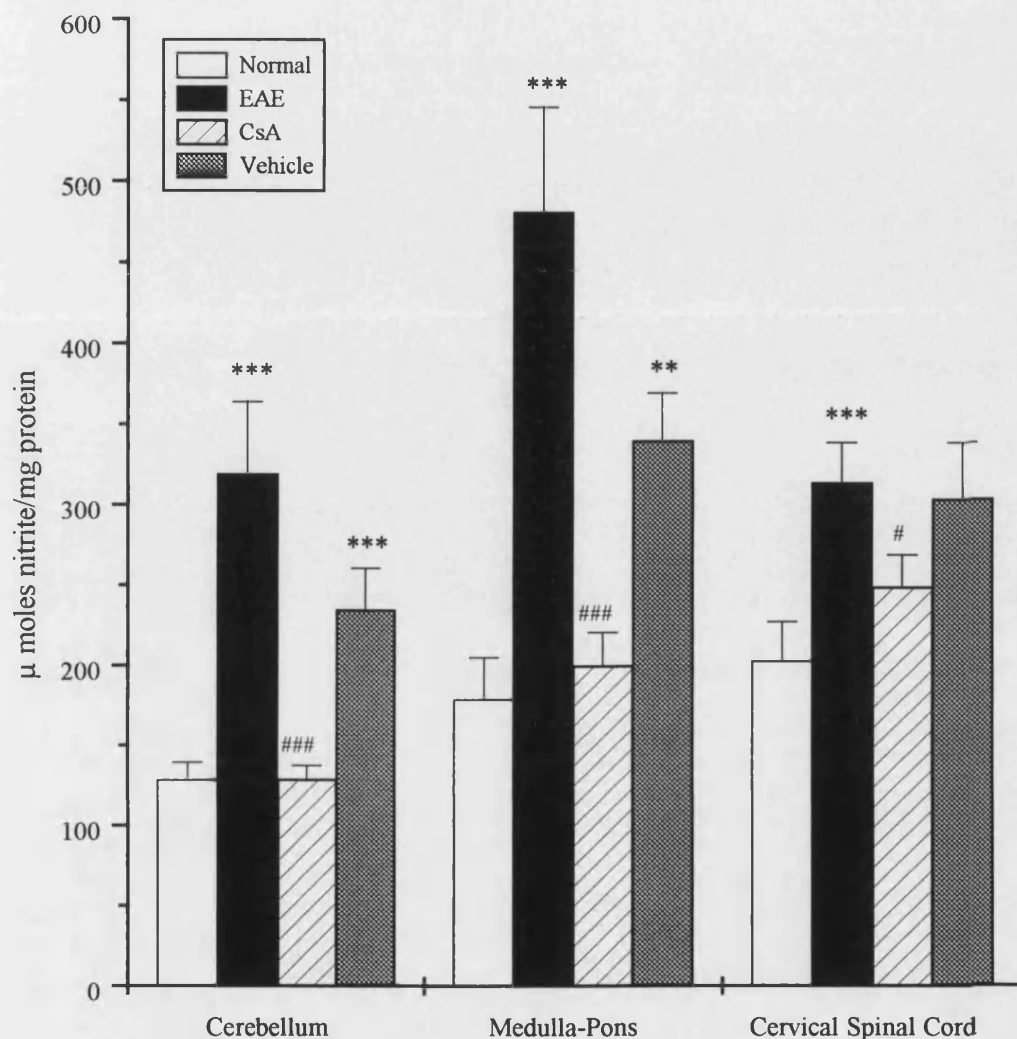


Figure 5.3 Effect of CsA administration on nitrite levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, $n = 12$; EAE-diseased Lewis rats, $n = 12$; EAE-sensitised Lewis rats orally dosed with CsA (50mg/kg body weight) once daily for 2 days from disease associated weight loss, $n = 8$; EAE-inoculated Lewis rats orally dosed with vehicle (extra virgin olive oil, 2ml/kg body weight) once daily for 2 days commencing from disease associated weight loss, $n = 8$. ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. # $p < 0.05$, ### $p < 0.001$ Mann Whitney U test compared to EAE animals.

techniques. iNOS protein levels were analysed semi-quantitatively and differences were expressed as a % change compared to normal levels.

5.3.1 Effect of Dex Administration on iNOS Protein Expression in CNS Cytosol

NOS protein bands were observed at 135 kDa in all CNS cytosol samples blotted with an anti-iNOS antibody. However, the detection method utilised differed depending on the CNS tissue used. Different detection methods were employed due to the variation in sample protein content which required the use of techniques with variable sensitivities. Immunoreactive bands were identified in cerebellum cytosol using the PAP complex (Figure 5.4) whereas iNOS protein was demonstrated in medulla-pons and cervical spinal cord preparations following ECL detection (Figure 5.5).

iNOS expression was elevated in CNS cytosol samples from EAE-inoculated animals (cerebellum 27 %, medulla-pons 142 %, cervical spinal cord 29 % above normal) (Figure 5.4 and 5.5). Administration of Dex reduced iNOS protein levels in CNS tissues from diseased rats (cerebellum 6 %, medulla-pons 31 %, cervical spinal cord 3 % above normal). However, vehicle treatment did not markedly affect CNS cytosol iNOS expression (cerebellum 44 %, medulla-pons 191 %, cervical spinal cord 29 % above normal) (Figure 5.4 and 5.5).

5.3.2 Effect of CsA Administration on iNOS Protein Expression in CNS Cytosol

EAE-diseased Lewis rats showed increased levels of iNOS protein at 135 kDa in all samples assayed (cerebellum 45 %, medulla-pons 161 %, cervical spinal cord 76 % above normal) (Figure 5.6 and 5.7). In contrast, decreased iNOS expression was observed in CNS cytosol preparations from EAE-sensitised animals administered CsA (cerebellum 1 %, medulla-pons 53 %, cervical spinal cord 7 % above normal). Following vehicle treatment no changes in iNOS protein levels were detected in diseased rats tissues (cerebellum 41 %, medulla-pons 137 %, cervical spinal cord 50 % above normal) (Figure 5.6 and 5.7).

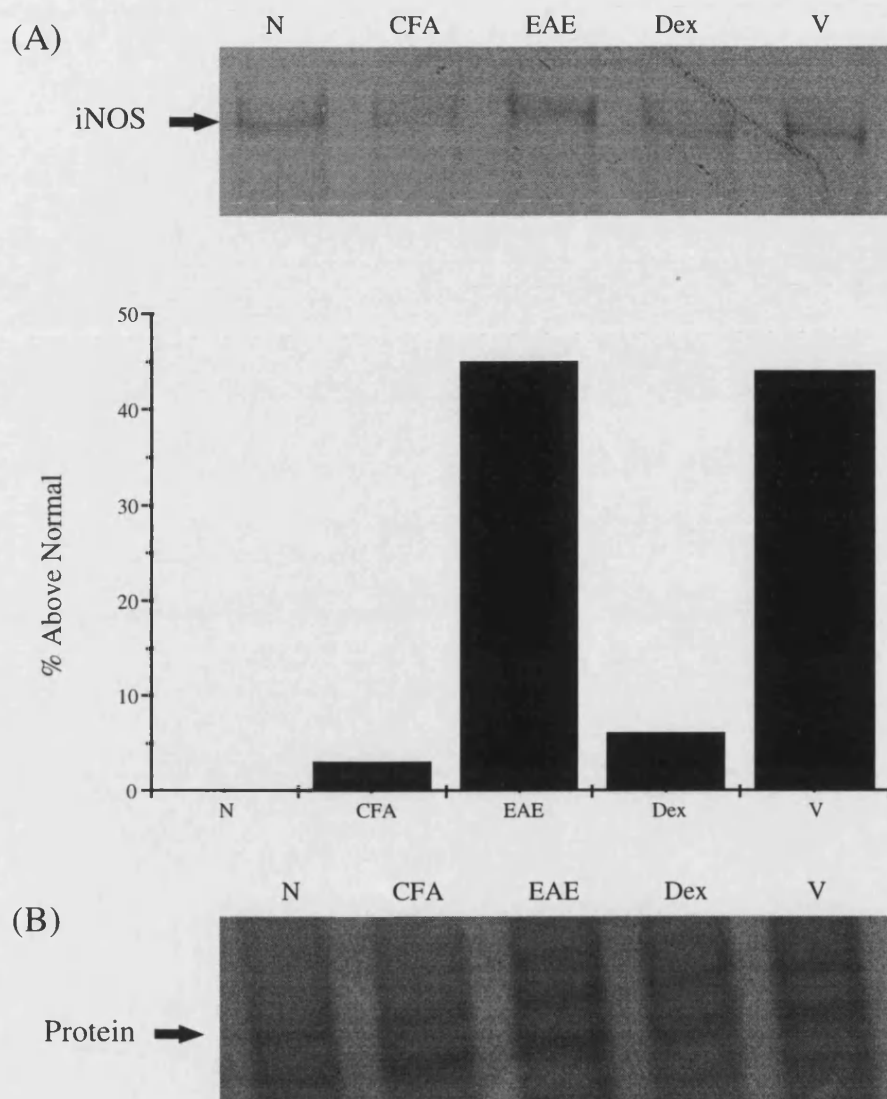


Figure 5.4 Effect of Dex administration on iNOS protein expression

CNS cytosol was obtained from the cerebellum of normal Lewis rats (N); CFA-inoculated rats (CFA); EAE-diseased animals (EAE) and EAE-inoculated rats injected s.c. with Dex (1 mg/kg body weight) or vehicle (PBS, 1 ml/kg body weight) twice daily for 2 days from disease associated weight loss. CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with anti-iNOS antibody. iNOS protein was detected using PAP complex (A). Densitometric analysis of blots was undertaken and following correction for differences in total protein content (B) iNOS levels were expressed as % above the normal value. Results shown are representative of 3 separate experiments.

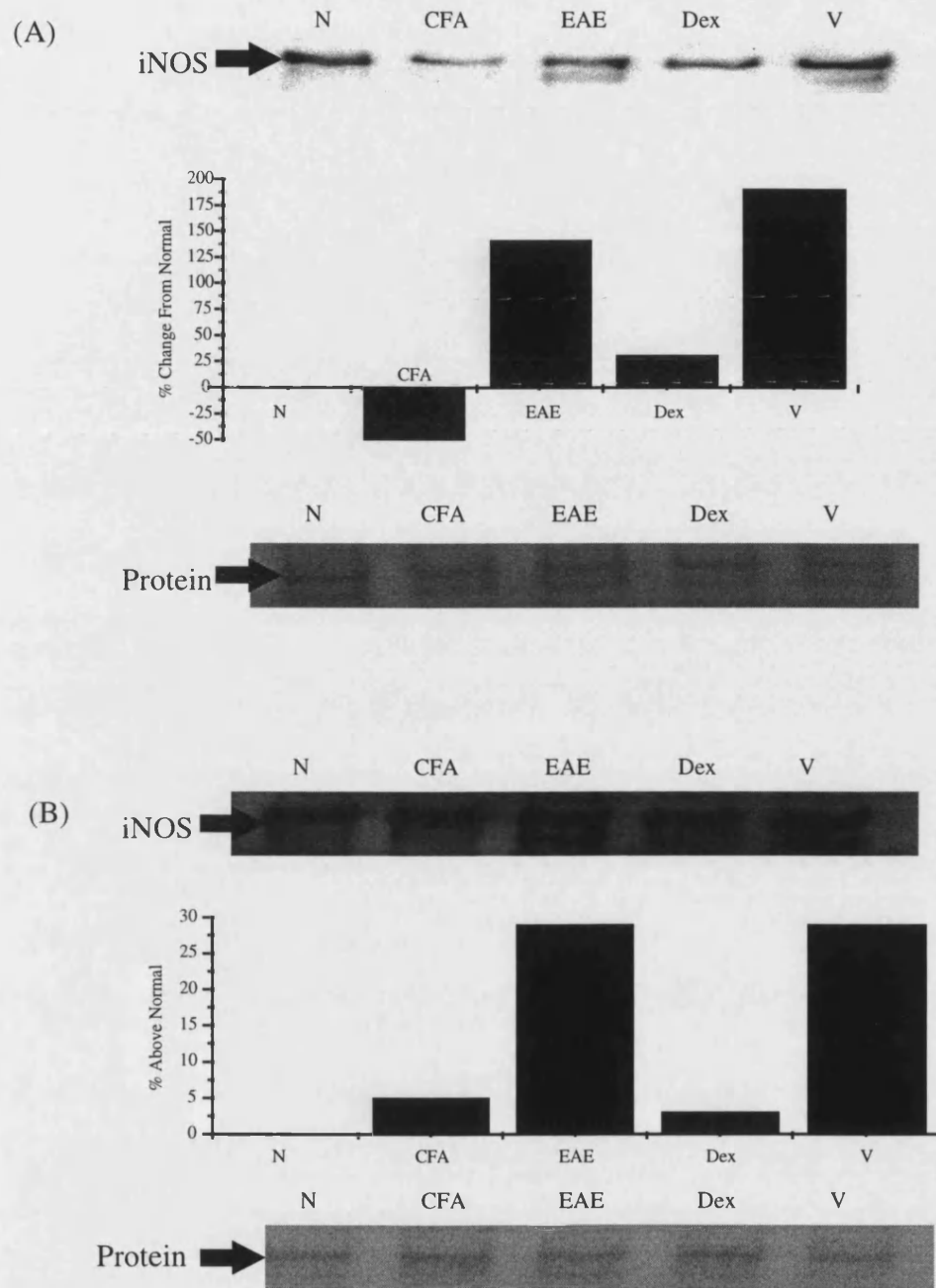


Figure 5.5 Effect of Dex administration on iNOS protein expression

CNS cytosol was obtained from (A) medulla-pons and (B) cervical spinal tissue of normal Lewis rats (N); CFA control inoculated Lewis rats (CFA); EAE-diseased animals (EAE); EAE-inoculated animals s.c. injected with Dex (1 mg/kg body weight) or vehicle (PBS, 1 ml/kg body weight) twice daily for 2 days from disease associated weight loss (Dex and V). Proteins were separated on SDS-gels and following electrophoretic transfer were blotted with anti-iNOS antibody. Specific protein was detected using ECL (top panel). Densitometric analysis of blots was undertaken and following correction for differences in total protein concentrations (bottom panel) results were expressed as a % change from the normal value. Results shown are representative of 3 separate experiments.

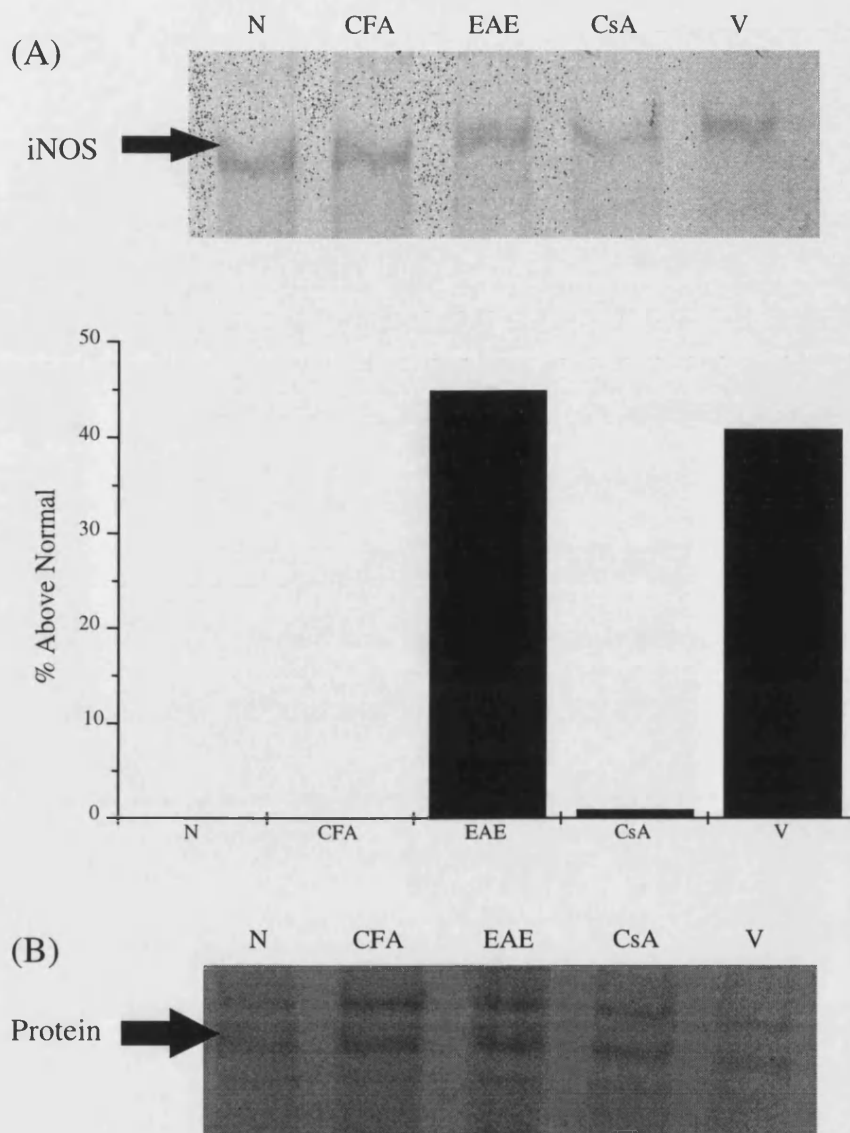


Figure 5.6 Effect of CsA administration on iNOS protein expression

CNS cytosol was obtained from the cerebellum of normal Lewis rats (N); CFA-inoculated rats (CFA); EAE-diseased animals (EAE); and EAE-sensitized Lewis rats orally dosed with CsA (50 mg/kg body weight) or vehicle (extra virgin olive oil, 2 ml/kg body weight) for 2 days commencing from disease associated weight loss (CsA and V). Proteins were separated on SDS-gels and following electrophoretic transfer they were blotted with anti-iNOS antibody. Specific protein was detected using PAP complex (A). Densitometric analysis of bands was undertaken and following correction for differences in total protein concentrations (B) iNOS levels were expressed as % above normal value. Results shown are representative of 3 separate experiments.

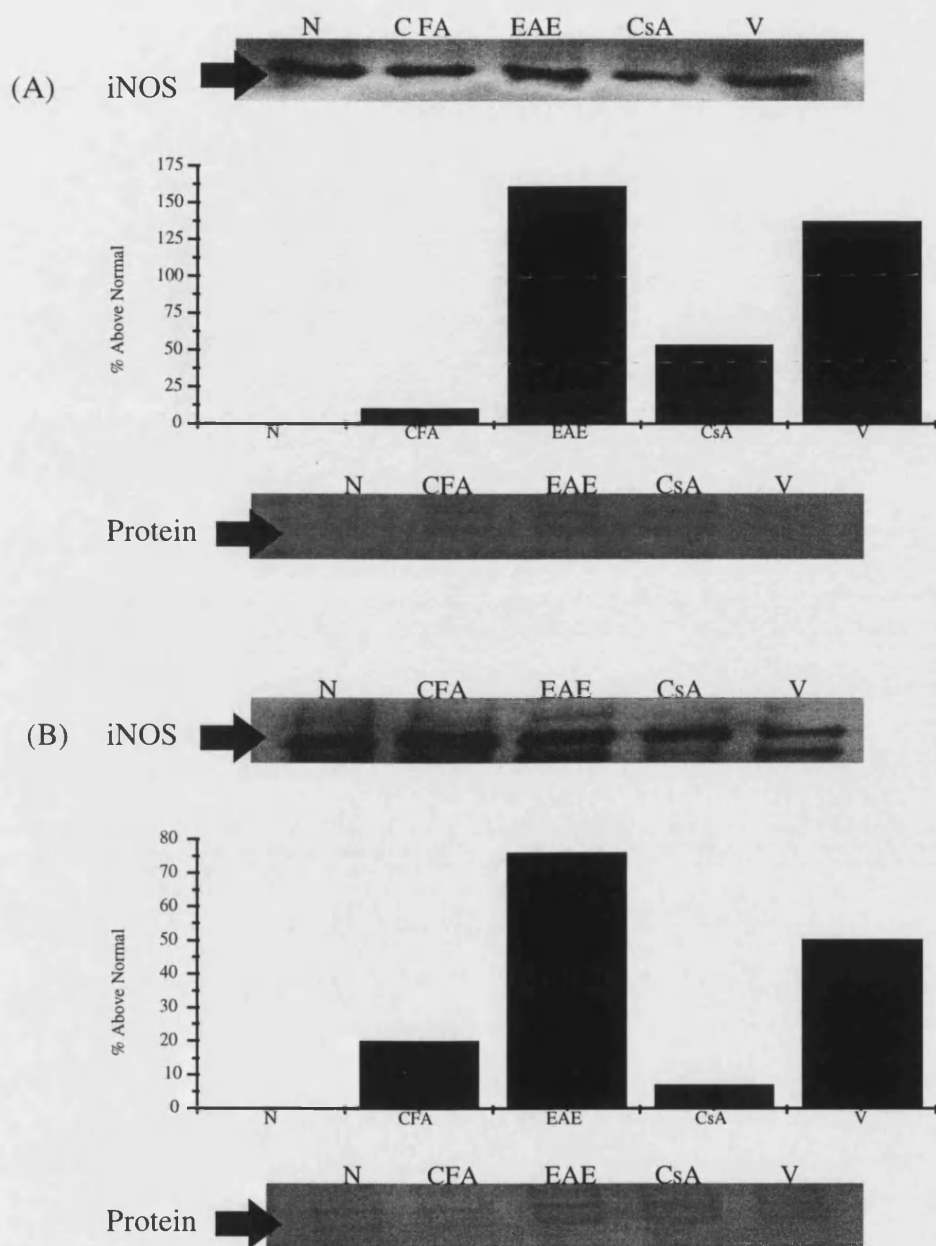


Figure 5.7 Effect of CsA administration on iNOS protein expression

CNS cytosol was obtained from (A) medulla-pons and (B) cervical spinal tissues of normal Lewis rats (N); CFA-inoculated rats (CFA); EAE-diseased animals (EAE); and EAE-sensitised Lewis rats orally dosed with CsA (50 mg/kg body weight) or vehicle (extra virgin olive oil, 2 ml/kg body weight) for 2 days commencing on disease associated weight loss (CsA and V). Cytosol proteins were separated on SDS-gels and following electrophoretic transfer they were blotted with anti-iNOS antibody. iNOS protein was detected using ECL (top panel). Densitometric analysis of bands was undertaken and following correction for differences in total protein concentrations (bottom panel) iNOS levels were expressed as % above normal value. Results shown are representative of 3 separate experiments.

5.4 Effect Of Immunosuppressive Agents On ROS Levels

Levels of superoxide and hydrogen peroxide were measured in CNS samples from EAE-sensitised Lewis rats treated with Dex or CsA to determine whether the immunosuppressive agents inhibit EAE-development through effects on ROS.

Superoxide levels were unaltered in the cerebellum, medulla-pons or cervical spinal cord of EAE-inoculated animals following administration of Dex (Figure 5.8a). Similarly, treatment of EAE-diseased rats with vehicle had no effect on the superoxide content of CNS samples analysed (Figure 5.8a).

Although cerebellar cytosol hydrogen peroxide levels were unchanged in Dex and vehicle dosed animals, hydrogen peroxide content of the medulla-pons and cervical spinal cord tissues were significantly elevated ($p < 0.05$) (Figure 5.8b).

CsA administration to EAE-sensitised rats had differential effects on ROS levels in CNS cytosol. Following treatment with CsA, superoxide levels were reduced in both the cerebella and cervical spinal cord cytosols of EAE-diseased rats (Figure 5.9a). However, no marked decrease in superoxide content was observed in the medulla-pons tissue of EAE-inoculated animals administered CsA. Moreover, vehicle treatment had a similar effect on CNS cytosol superoxide levels (Figure 5.9a).

Hydrogen peroxide levels were also decreased in the cerebella of EAE-sensitised Lewis rats dosed with CsA (Figure 5.9b). In contrast, no significant reduction in hydrogen peroxide was detected in medulla-pons or cervical spinal cord cytosol fractions from CsA treated animals (Figure 5.9b). Furthermore, vehicle administration had comparable effects on CNS cytosol levels of hydrogen peroxide (Figure 5.9b).

5.5 Effect of Immunosuppressive Agents on Cellular Infiltration

The ability of immunosuppressive agents to prevent inflammatory cell infiltration during EAE was assessed by determining the number of perivascular infiltrates in cervical spinal cord sections (Figure 5.10). Administration of both Dex and CsA significantly reduced cellular migration into cervical spinal cords of diseased animals ($p < 0.01$). However, vehicle treatment had no effect on cell infiltration (Table 5.2).

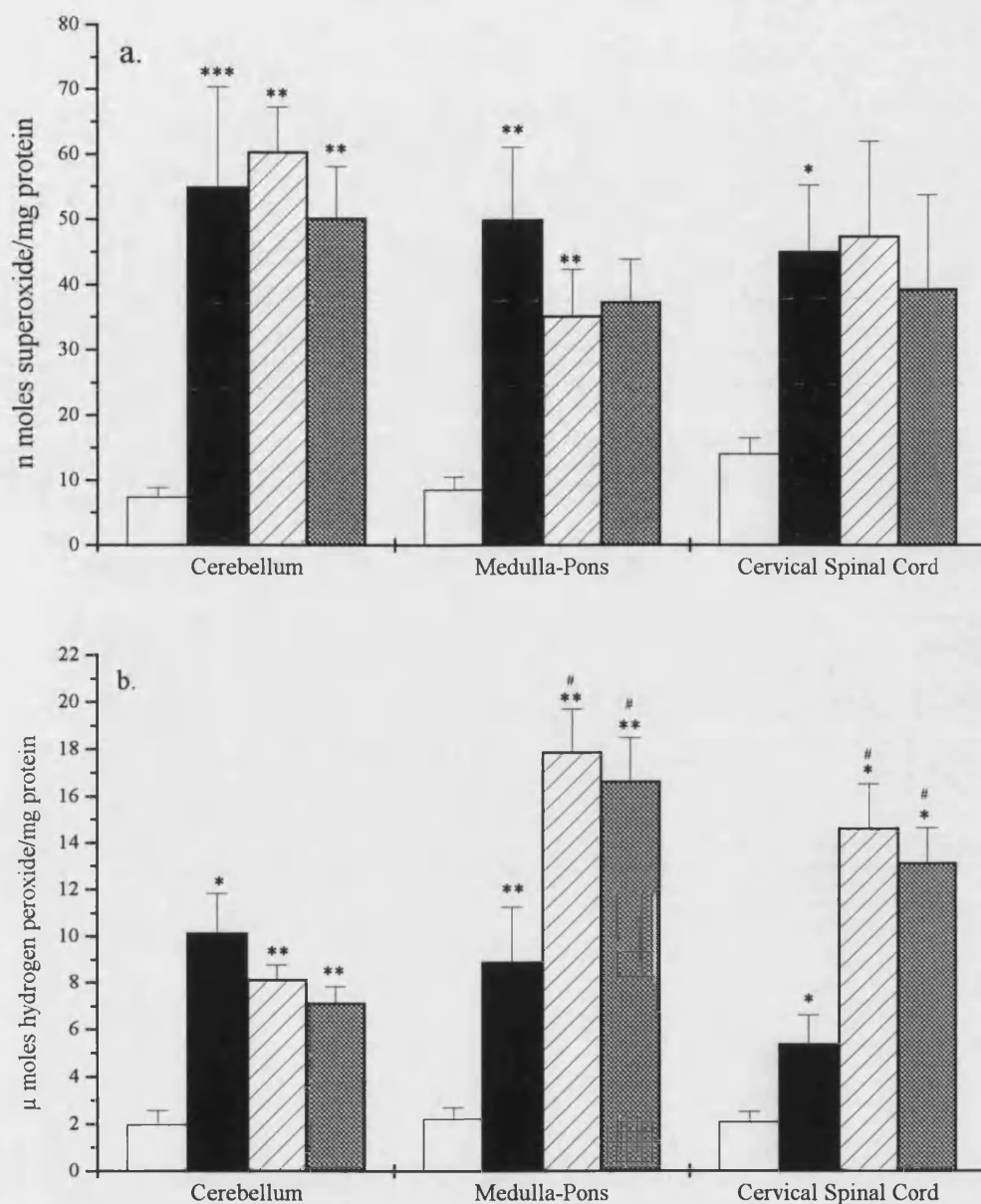


Figure 5.8 Effect of Dex administration on ROS levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;
 ▨ EAE-sensitised Lewis rats s.c. injected with Dex (1mg/kg body weight) twice daily for 2 days from disease associated weight loss, n = 5; ▩ EAE-inoculated Lewis rats s.c. dosed with vehicle (PBS, 1ml/kg body weight) twice daily for 2 days commencing from disease associated weight loss, n = 5.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. # $p < 0.05$ Mann Whitney U test compared to EAE animals.

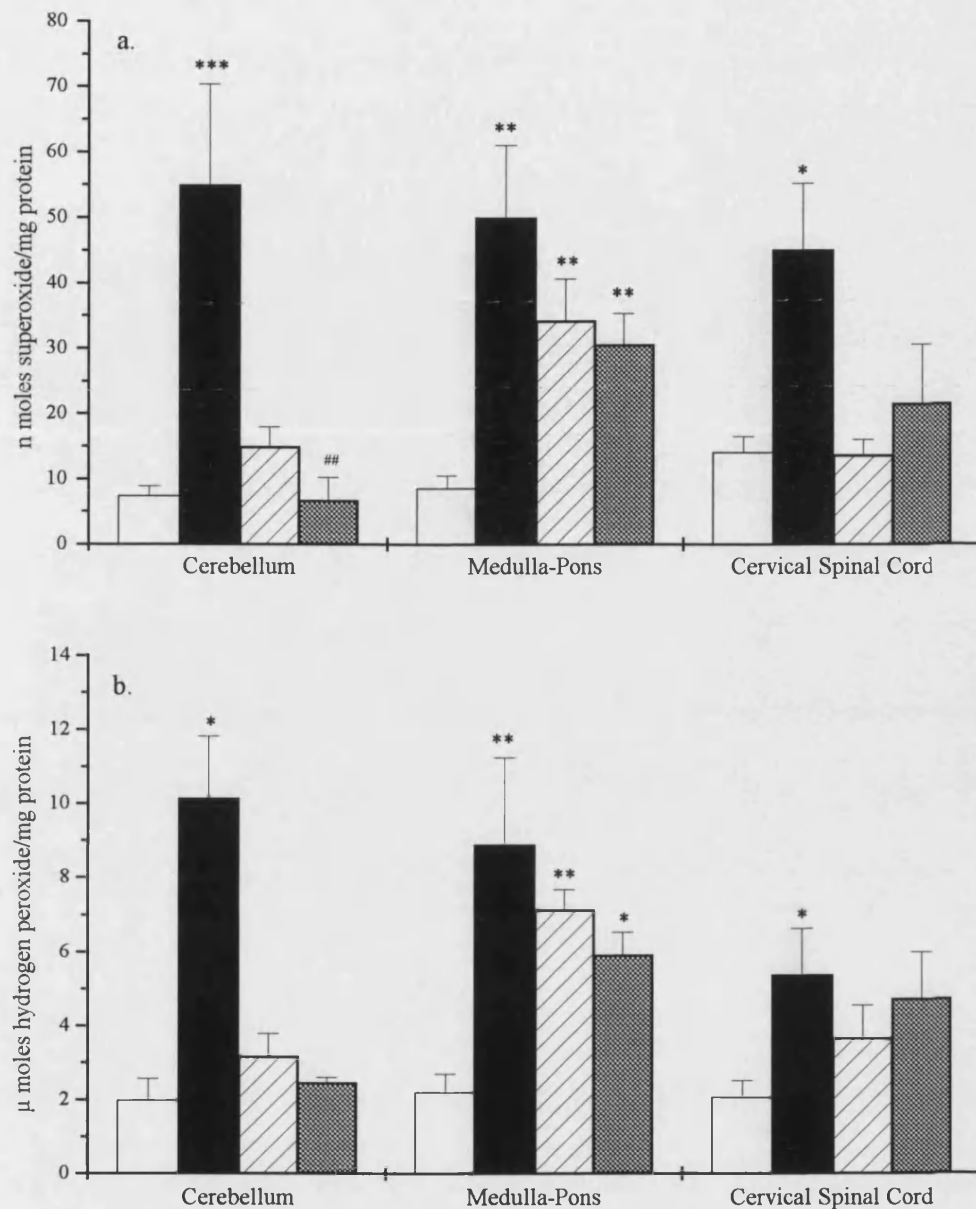


Figure 5.9 Effect of CsA administration on ROS levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;

▨ EAE-sensitised Lewis rats orally dosed with CsA (50mg/kg body weight) once daily for 2 days from disease associated weight loss, n = 5;

▤ EAE-inoculated Lewis rats orally dosed with vehicle (extra virgin olive oil, 2ml/kg body weight) once daily for 2 days commencing from disease associated weight loss, n = 5. *p<0.05, **p<0.01, ***p<0.001

Mann Whitney U test compared to normal animals. ##p<0.01 Mann Whitney U test compared to EAE animals.

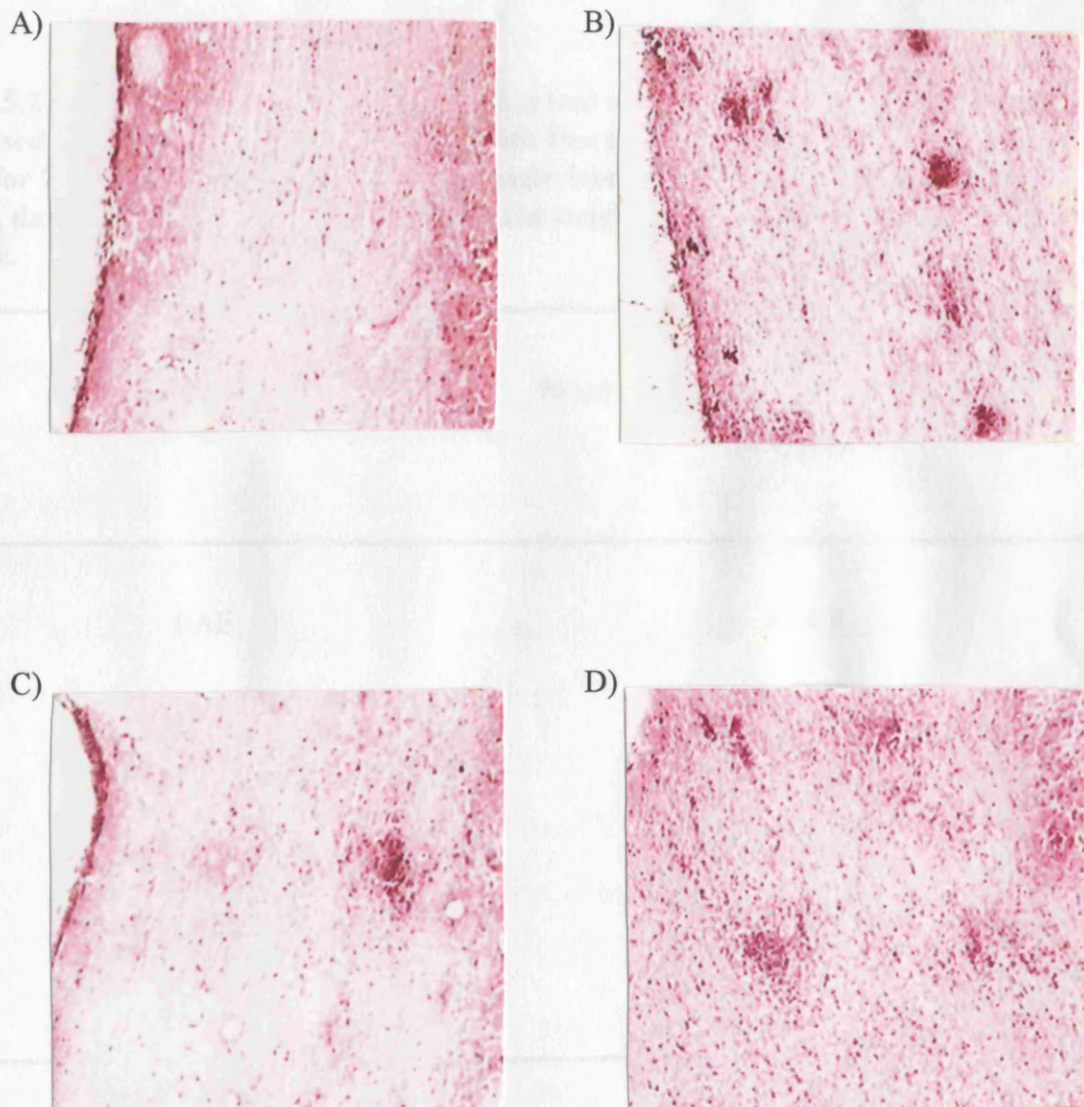


Figure 5.10 Effect of immunosuppressive agents on cellular infiltration during EAE

Cellular infiltration in haematoxylin and eosin stained cervical spinal cord samples from EAE-inoculated animals following treatment with A) Dex (1 mg/kg body weight, s.c., twice daily for 2 days from disease associated weight loss), B) PBS vehicle (1 ml/ kg body weight, s.c., twice daily for 2 days from disease associated weight loss), C) CsA (50 mg/kg body weight, orally, daily for 2 days from disease associated weight loss) and D) Extra virgin olive oil (1 ml/ mg/kg body weight, orally, daily for 2 days from disease associated weight loss). Magnification x40.

Table 5.2 Perivascular infiltrates in cervical spinal cord samples from EAE-sensitised Lewis rats following treatment with Dex (1 mg/kg body weight, s.c., twice daily for 2 days from disease associated weight loss), CsA (50 mg/kg body weight, orally, daily for 2 days from disease associated weight loss) or the appropriate vehicle.

Group	Mean Number of Lesions ^a /10 mm Section (± S.E.M.)
EAE	53 ± 4
Dex	11 ± 1*** ^{††}
Vehicle	47 ± 4
CsA	24 ± 1*** ^{††}
Vehicle	37 ± 3

^a Sections were cut at one standard depth, stained with haematoxylin and eosin and the total number of lesions per section was determined using light microscopy (x40). Values are means of 18 sections from 3 animals ± S.E.M. ***p<0.001 Mann Whitney U test compared to EAE group. ^{††}p<0.01, ^{†††}p<0.001 Mann Whitney U test compared to vehicle group.

5. DISCUSSION

The immunosuppressive actions of Dex and CsA during EAE will be considered with respect to modulation of NO production, ROS generation and other anti-inflammatory effects.

5.1 Immunosuppression of EAE

5.1.1 Glucocorticoids

Many studies have demonstrated the inhibitory effect of prophylactic or therapeutic steroid treatment on EAE disease severity (Komarek & Dietrich 1971, Levine & Sowinski 1980, Bolton & Flower 1989, Desari & Barton 1989). In addition to actions on EAE, high-dose steroid therapy has been effectively utilised for the management of MS (Durelli *et al.* 1986, Troiano *et al.* 1987, Compston 1988, Milanese *et al.* 1989, Beck *et al.* 1993, Kupersmith *et al.* 1994, La Mantia *et al.* 1994). In this study therapeutic administration of the glucocorticoid, dexamethasone (Dex), dramatically reduced the development of neurological EAE. These results are in agreement with data obtained previously (Paul & Bolton 1995).

Endogenous glucocorticoids also have a marked effect on EAE development. Apparently insignificant stressors such as animal handling have been found to elevate corticosterone levels and ameliorate EAE (Levine *et al.* 1962). Moreover endogenous steroids are thought to be associated with the spontaneous recovery detected in EAE-diseased rats and mice (Levine *et al.* 1962, Mackenzie *et al.* 1989, Bolton *et al.* in press). Corticosteroid levels are observed to increase 10 fold during the expression of peak disease symptoms but fall to normal levels when signs of EAE resolve (MacPhee *et al.* 1989). Further evidence for the important role played by endogenous corticosteroids is provided by work demonstrating that adrenalectomy or treatment with the steroid

receptor antagonist, RU38486, markedly exacerbates neurological EAE (Levine *et al.* 1962, Bolton & Flower 1989, MacPhee *et al.* 1989).

5.1.2 CsA

Several studies have established that the immunomodulatory agent, CsA suppresses both actively induced (Bolton *et al.* 1982b, Hinrichs *et al.* 1983, Mustafa *et al.* 1993, Branisteanu *et al.* 1995) and adoptively transferred EAE (Bolton *et al.* 1982a, Bolton 1992). Furthermore, CsA has been utilised in the treatment of chronic relapsing forms of the disease (Feurer *et al.* 1988, Kovarik *et al.* 1995). High dose CsA has also been used in the management of MS (Rudge *et al.* 1989, Ruutinen *et al.* 1991), but the effectiveness of treatment has been associated with toxic side effects. The results of the present study reconfirm that administration of CsA delays the onset and decreases the severity of neurological deficits in EAE.

5.2 Immunosuppression of NO

5.2.1 Glucocorticoids

Corticosteroids have many potent anti-inflammatory and immunosuppressive effects (Figure 5.11), some of which may be important in EAE modulation following Dex treatment. For example, glucocorticoids have previously been shown to prevent NO synthesis by inducible isoforms of NOS (Di Rosa *et al.* 1990, Radomski *et al.* 1990, Brenner *et al.* 1994). Glucocorticoids have been shown to inhibit NO production at the level of enzyme expression, activity and effector function (Figure 5.12). As neurological development of EAE was characterised by an elevation in CNS levels of iNOS protein and nitrite, Dex administration may inhibit NO formation during EAE and so reduce disease severity. Interestingly nitrite levels in the cerebellum and medulla-pons tissues of EAE-diseased rats were decreased by the administration of Dex.

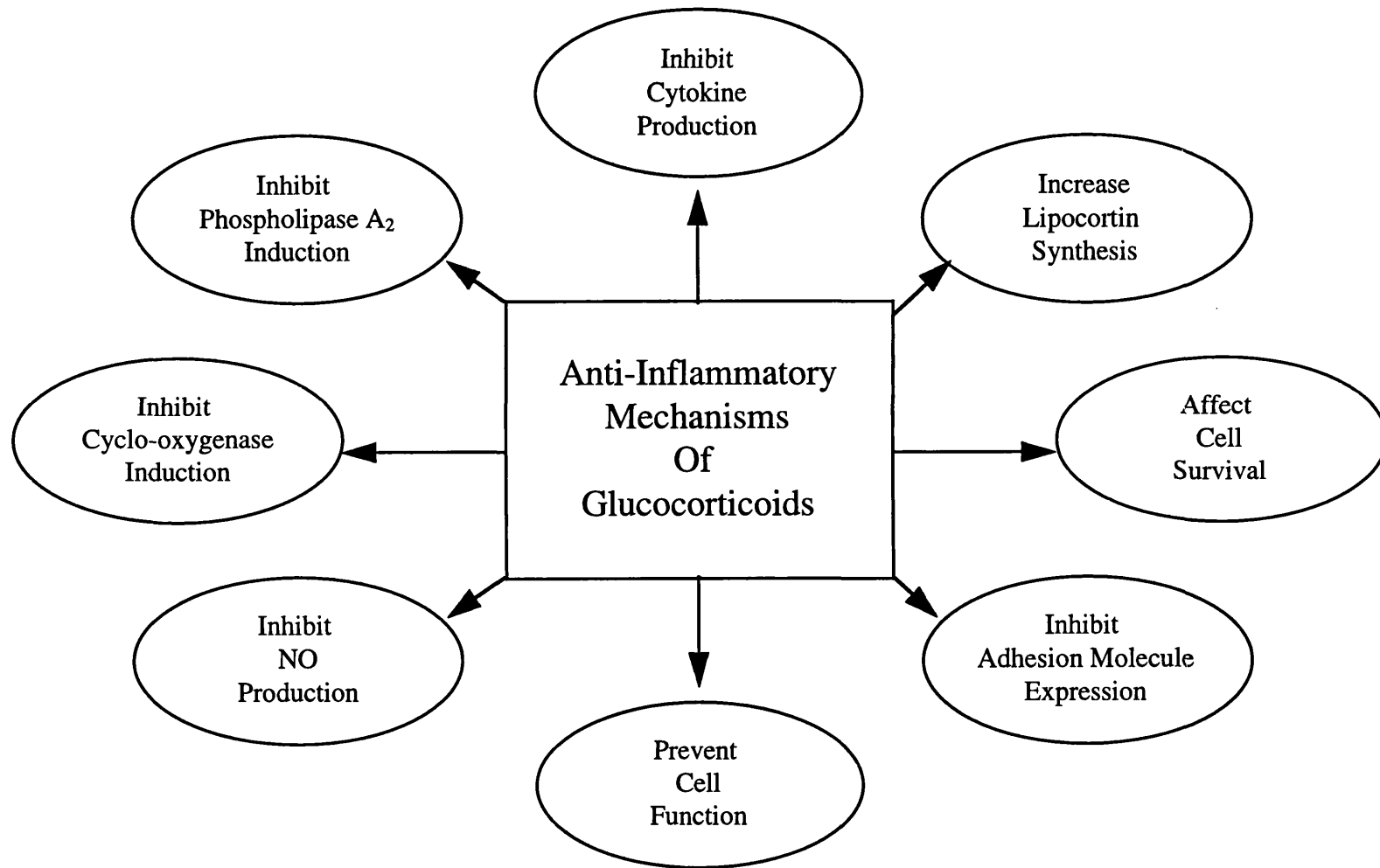


Figure 5.11 Summary of anti-inflammatory mechanisms of glucocorticoids.

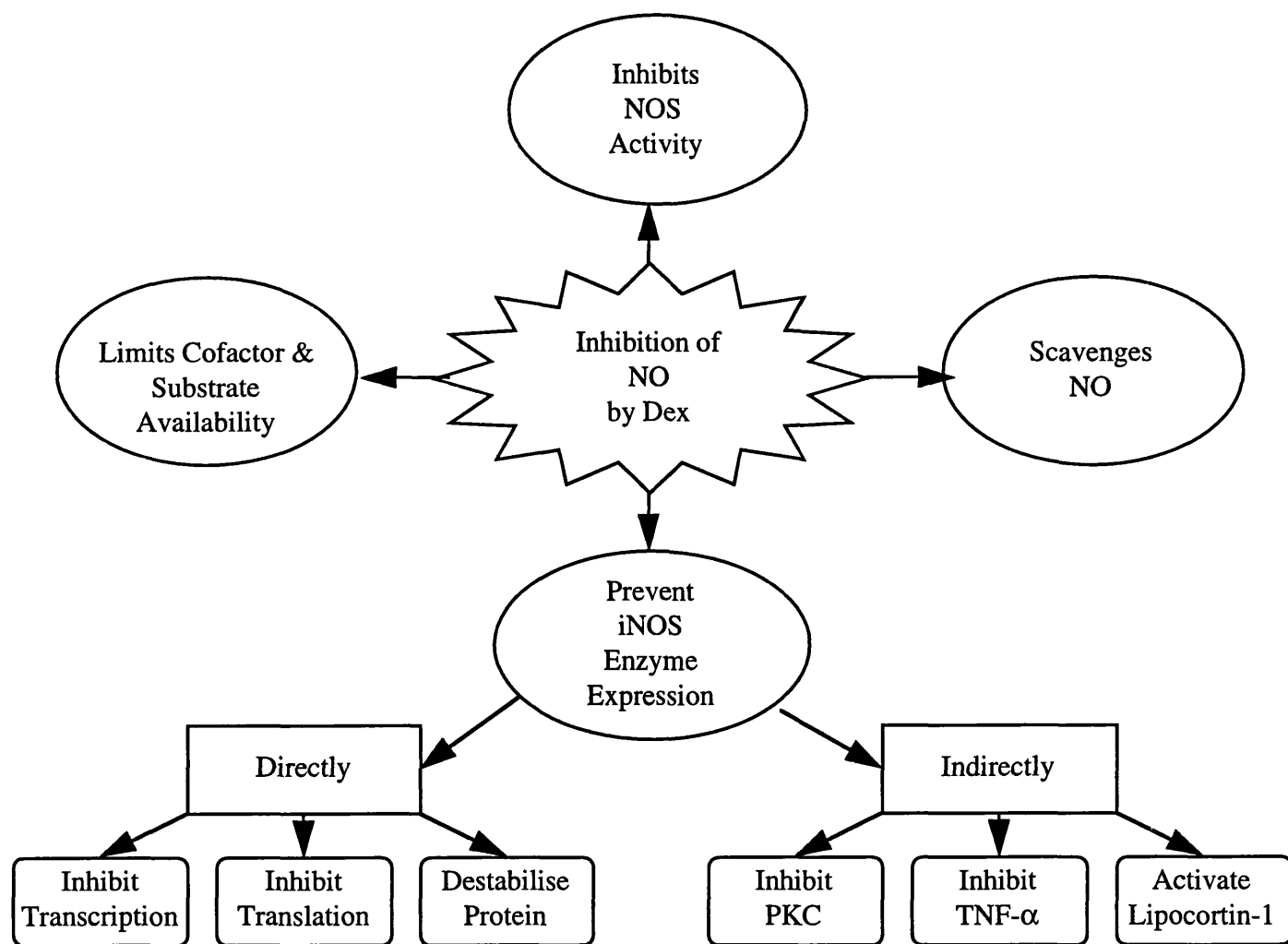


Figure 5.12 Summary of mechanisms of NO inhibition by Dex.

5.2.1.1 Inhibition of iNOS Expression

Dex prevents NO formation by inhibiting the expression of iNOS (Rees *et al.* 1990a, McCall *et al.* 1991b, O'Connor & Moncada 1991, Palmer *et al.* 1992, Baydoun *et al.* 1993, Haddad *et al.* 1995). However, the site of action at which Dex prevents iNOS induction is unknown. Dex may inhibit the induction of NOS directly or indirectly by preventing the synthesis or activity of cytokines, such as TNF- α , which are responsible for induction of the enzyme

5.2.1.1a Direct Effects.

Direct inhibition of NOS expression by glucocorticoids can occur at the level of gene transcription or translation (Kunz *et al.* 1996). Dex was observed to prevent the induction of mRNA for iNOS (Geller *et al.* 1993b). However, the molecular mechanism of this effect has not been elucidated. Dex may inhibit gene expression by binding to the regulatory element in the 5' flanking region of target genes and thereby block gene transcription (Beato 1989, Barnes & Adcock 1993). Alternatively, interactions between Dex and other transcription factors such as NF- κ B (Mukaida *et al.* 1994, Van de Stolpe *et al.* 1994, Kleinert *et al.* 1996) or AP-1 (Jonat *et al.* 1990) have been suggested to mediate gene repression by glucocorticoids. In addition, Dex may exert post-transcriptional effects on the synthesis and stability of iNOS (Kunz *et al.* 1996).

5.2.1.1b Indirect Effects

Indirect actions of Dex to inhibit NO formation may proceed via several mechanisms. For example, corticosteroids may prevent PKC-mediated NO production (Neary *et al.* 1988). Indeed, Dex inhibits PKC activity with a concomitant reduction in NO (Jun *et al.* 1994). Glucocorticoids may prevent NO formation via an inhibition of TNF- α (Brenner *et al.* 1993) which has been identified as a principal cytokine involved in iNOS induction (Nathan 1992). Therefore, TNF- α inhibition by Dex may indirectly decrease NO production. Finally work suggests the inhibition of NOS by Dex may be mediated via lipocortin-1 (Wu *et al.* 1995).

5.2.1.2 Possible Actions in EAE

In the present investigation iNOS protein was markedly reduced in CNS tissues from EAE-sensitised Lewis rats administered Dex. Therefore, Dex may function to prevent the transcription or translation of iNOS during disease. iNOS has been implicated in EAE aetiology (Koprowski *et al.* 1993), and the protective effects of Dex treatment may be mediated, at least in part, via iNOS inhibition. Dex suppression of iNOS during EAE may occur through direct effects on protein transcription or translation. In addition, steroid treatment may indirectly affect iNOS as a consequence of a decrease in CNS levels of TNF- α . TNF- α has been implicated in the pathogenesis of EAE (Brosnan *et al.* 1988, Selmaj *et al.* 1988, Powell *et al.* 1990, Kuroda & Shimamoto 1991) and one of the effector mechanisms may be the induction of iNOS. Therefore Dex administration to EAE-diseased rats may reduce TNF- α and concomitantly decrease iNOS expression.

5.2.1.3 Other Inhibitory Effects

In addition to effects on enzyme expression Dex may directly inhibit NOS activity or scavenge NO to cause a reduction in nitrite levels (Cetkovic-Cvrlje *et al.* 1993). Moreover recent reports have suggested that glucocorticoids regulate iNOS through actions on enzyme cofactor and substrate availability (Simmons *et al.* 1996). The decrease in CNS nitrite levels observed following Dex administration to EAE-diseased rats may occur as a consequence of limited co-factor availability, direct inhibition of NOS or scavenging of NO. However, further work would be required to confirm if any of these mechanisms are occurring.

5.2.1.4 Upregulation of cNOS

Although Dex prevents the induction of iNOS, it has also been demonstrated to upregulate cNOS-dependent NO formation (Baltrons *et al.* 1995). Furthermore, Kiel *et al.* (1995) have reported that *in vivo* administration of Dex does not alter *ex vivo* nitrite levels. Therefore, under certain circumstances Dex does not inhibit NO production. Interestingly, in the present study, no reduction in cervical spinal cord nitrite levels were

detected in glucocorticoid treated animals. However a reduction in iNOS protein expression was observed in the sample tissue. This observation may be accounted for by the ability of Dex to increase the activity of other NOS enzymes and so overcome the reduction in NO produced by iNOS. However, enzyme activity measurements would need to be undertaken to validate the hypothesis.

5.2.2 CsA

Although the immunosuppressive effects of CsA on EAE appear to be mediated primarily via T cell modification (Klaus 1981, Killen & Swanborg 1982) other immunomodulatory mechanisms may be involved. Indeed CsA has been shown to prevent NO production (Mühl *et al.* 1993, Conde *et al.* 1995, Kunz *et al.* 1995, Marunmo *et al.* 1995). Similarly immunomodulation of EAE by CsA may proceed via NO inhibition as nitrite levels were decreased in the CNS of drug treated animals.

CsA may prevent NO formation through effects on the expression and activity of iNOS (Conde *et al.* 1995, Hattori & Nakaisha 1995). In contrast to inhibition of NO by Dex, CsA has not been identified as limiting co-factor and substrate availability (Hattori & Nakaisha 1995) nor has the drug been shown to directly scavenge NO. Inhibition of NOS expression by CsA is reported to be mediated via similar effector mechanisms to those elucidated for iNOS downregulation by Dex. Therefore, CsA affects iNOS expression, at least in part, at the level of transcription (Mühl *et al.* 1993, Marumoto *et al.* 1995). Furthermore, NOS induction is prevented by CsA inhibiting the activation of transcription factors such as NF- κ B (Kunz *et al.* 1995, Marumoto *et al.* 1995). Additionally CsA may directly inhibit NOS activity by binding to the calmodulin site on the enzyme resulting in inactivation (Conde *et al.* 1995).

In the present study CsA was observed to decrease iNOS protein expression in CNS tissue from EAE-sensitised rats. The results suggest that the reduction in CNS nitrite levels detected in drug treated animals may result from an inhibition of iNOS expression. However as the effects of CsA on iNOS activity in diseased rats were not

assessed, the decrease in CNS nitrite content may also arise from a reduction in enzyme activity.

5.3 Immunosuppression of ROS

5.3.1 Glucocorticoids

Although glucocorticoid effects on ROS production have been extensively examined, contradictory results have been reported. *In vitro* studies have established inhibitory (Rist & Naftalin 1993, Rosnel *et al.* 1995), stimulatory (Haar & Nielson 1988) or no effect (Dieter *et al.* 1986, Schaffner & Schaffner 1987, Cox 1995) of Dex on ROS production. *In vivo* data demonstrating the effect of glucocorticoids is scant, however, superoxide anion production in peritoneal inflammatory exudates was unaltered following corticosterone administration (Fleming *et al.* 1990).

The results of the present study indicate that Dex treatment did not alter CNS superoxide levels in EAE-diseased rats and reconfirm the previous *in vivo* observations of Fleming *et al.* (1990). However, hydrogen peroxide levels were significantly elevated in medulla-pons and cervical spinal tissue of EAE-sensitised animals administered Dex. Similar results were observed in vehicle treated rats suggesting a non-specific effect on hydrogen peroxide. Therefore, the results of this investigation implies Dex does not exert immunosuppressive effects on the development of EAE via ROS modulation.

5.3.2 CsA

CsA has been shown to inhibit the production of ROS in human neutrophils and murine macrophages (Chiara *et al.* 1989, Wenzel-Seifert *et al.* 1991, Kurokawa *et al.* 1992). However, differential effects of CsA have been observed on ROS generation in resting and activated macrophages (Chiara & Sobrino 1991). Furthermore, the present data demonstrate a disparity in ROS levels in CNS tissues of EAE-diseased animals following CsA administration. Whereas both superoxide and hydrogen peroxide levels were reduced in cerebellar tissue of drug treated rats, only the superoxide content was

decreased in cervical spinal cord following administration of CsA. Moreover, drug treatment did not alter superoxide or hydrogen peroxide content in other CNS areas examined which may be accounted for by the ability of CsA to prevent ROS production from resting but not activated macrophages (Chiara & Sobrino 1991). However, the decrease in ROS levels following drug administration may be due to non-specific effects as similar inhibition was observed upon vehicle treatment. Therefore, CsA does not appear to exert immunosuppressive effects on EAE by altering CNS levels of ROS.

5.4 Additional Immunosuppressive Actions

5.4.1 Glucocorticoids

In addition to effects on NO production Dex administration may prevent EAE development through inhibitory actions on several other important inflammatory mediators (Hartung *et al.* 1992, Barnes 1995) (Figure 5.11). For example, corticosteroids have been shown to prevent the transcription of several cytokines such as IL-1, IL-6, TNF- α and IFN- γ which have been implicated in the pathogenesis of EAE (Laurenzi *et al.* 1990, Mustafa *et al.* 1991, Merrill *et al.* 1992, Willenborg 1995). Furthermore, glucocorticoids inhibit the induction of genes coding for cyclooxygenase and phospholipase A₂, both of which may play a role in EAE (Wecker *et al.* 1991).

5.4.1.1 Adhesion Molecules

Another group of molecules thought to be important in EAE pathogenesis which are inhibited by Dex are the adhesion molecules (Cannella *et al.* 1991, Archelos *et al.* 1993, Willenborg *et al.* 1993). Adhesion molecules play a key role in the trafficking of inflammatory cells into the CNS during EAE (Raine *et al.* 1990, O'Neil *et al.* 1991). However adhesion molecule expression can be reduced by corticosteroids. Glucocorticoids can directly inhibit the expression of adhesion molecules at the level of gene transcription (Cronsterin *et al.* 1992, Aziz & Wakefield 1996). Furthermore, steroids may indirectly prevent expression via inhibitory effects on cytokines such as IL-1

and TNF- α which invariably upregulate the expression of adhesion molecules (Hughes *et al.* 1988). As a consequence of reducing adhesion molecule expression, infiltration of inflammatory cells into the CNS may be prevented. Indeed, the number of perivascular infiltrates within the spinal cord of EAE-diseased animals was significantly decreased following Dex treatment. Hence Dex administration may inhibit neurological EAE by preventing the CNS infiltration of immunocompetent cells.

5.4.1.2 BBB Breakdown

Additionally, Dex may prevent EAE through an inhibition of BBB breakdown. Disruption of the BBB has been identified as a fundamental event in the development of EAE. Furthermore, Dex administration has been shown to reduce the cerebrovascular permeability associated with EAE (Paul & Bolton 1995). Such BBB permeability alterations may subsequently restrict the passage of immunocompetent cells into the CNS and so prevent the neurological development of disease.

5.4.1.3 T cells

Corticosteroids may exert a longer lasting effect to suppress the development of EAE by altering the cytokine profile of CD4⁺ T lymphocytes responsible for disease induction (Ramirez *et al.* 1996). Dex administration can induce a pattern of cytokine production characteristic of a Th2 response. Levels of IL-4, IL-10 and IL-13 mRNA were observed to increase following Dex treatment whereas IFN- γ and TNF- α synthesis were decreased. Although the mechanisms responsible for the changes in cytokine expression are unknown it is possible that corticosteroids induce the differentiation of a Th2 response in CD4⁺ T cells, or that differential expansion of resident Th2 cells occurs in the presence of Dex (Ramirez *et al.* 1996).

5.4.1.4 Lipocortins

The immunosuppressive effects of Dex during EAE may be mediated by the CNS induction of anti-inflammatory effector proteins termed lipocortins (Flower 1988). Lipocortins have previously been identified in the CNS of EAE-diseased animals (Bolton *et al.* 1990) and later studies proposed that lipocortins may be involved in typical corticosteroid-induced recovery from EAE (Elderfield *et al.* 1993). Therefore, in this study Dex administration may aid in the prevention of EAE by enhancing lipocortin production.

5.4.2 CsA

The immunosuppressive properties of CsA can be largely attributed to the inhibition of T cell activation (Borel *et al.* 1976, Cantoni *et al.* 1994) (Figure 5.13). Indeed CsA interferes with the activity of a Ca^{2+} -regulated signalling pathway in T lymphocytes (Morris 1994, Mattila 1996). T cells are vital for the induction of EAE. Therefore the drug may prevent neurological disease development by inhibiting T cell activation and proliferation. In addition, CsA may prevent EAE by increasing T lymphocyte production of IL-13 (Van der Pouw Kraan *et al.* 1996). IL-13 is an immunomodulatory cytokine which has previously been demonstrated to exert inhibitory effects in EAE (Cash *et al.* 1994). CsA may also mediate its effects on EAE via an inhibition of adhesion molecules (Molossi *et al.* 1995). A decrease in adhesion molecule expression may result in reduced CNS inflammatory cell infiltration. Furthermore, drug administration caused a significant reduction in perivascular infiltrates in the spinal cord of EAE-sensitised Lewis rats. Therefore, CsA may alter neurological EAE by preventing the CNS influx of immunocompetent cells. Inflammatory cell infiltration and subsequent disease development may also be inhibited through direct drug actions on the permeability of the BBB. Indeed, CsA administration has previously been observed to reduce neurovascular disruption during EAE and suppress disease development (Paul & Bolton 1995).

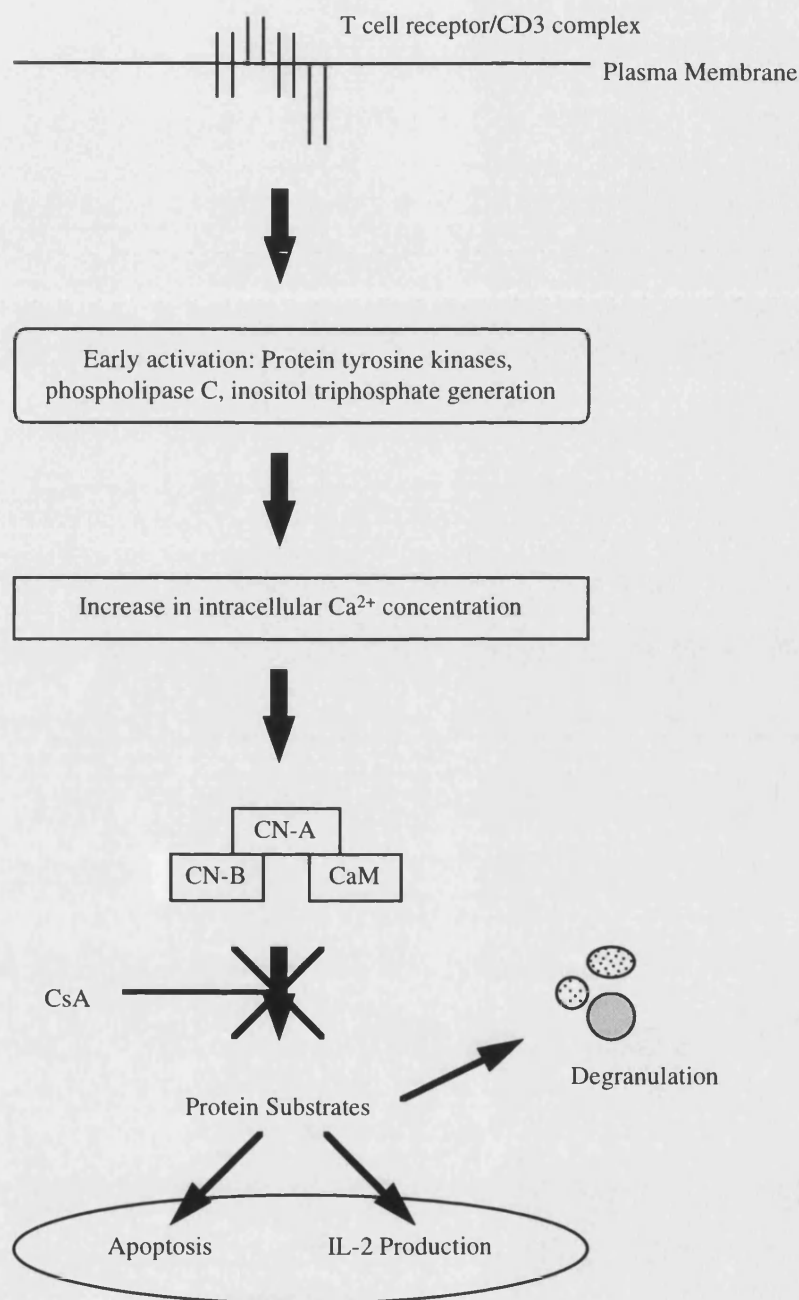


Figure 5.13 Inhibition of T cell responses by CsA

Stimulation via the T cell receptor/CD3 complex induced activation of protein tyrosine kinases and phospholipase C, leading to the generation of inositol triphosphate. (IP_3). The IP_3 release increases the concentration of intracellular Ca^{2+} which activates calmodulin (CaM) and CaM-dependent enzymes. The Ca^{2+} - and CaM-dependent phosphatase, calcineurin (CN) is a direct target of CsA. CN is involved in several T lymphocyte activation responses via dephosphorylation of critical phosphoprotein substrates. Therefore, the binding of CsA to CN prevents T cell activation. (Modified from Fruman *et al.* 1994)

CHAPTER 6

NOS INHIBITORS

6. RESULTS

As established neurological EAE was characterised by a significant elevation in CNS cytosol nitrite levels, NOS inhibitors were administered to EAE-inoculated Lewis rats to determine the effect that reducing NO would have on EAE development. Animals sensitised for EAE were treated with one of 3 different NOS inhibitors. Each NOS inhibitor employed has varying selectivity for the 3 NOS isoforms. L-NAME was utilised as a non-selective inhibitor of both c and iNOS whereas 7-NI and AG were used to selectively inhibit b and iNOS respectively (Rees *et al.* 1990b, Griffiths *et al.* 1993, Moore *et al.* 1993).

6.1 Effect of NOS Inhibitors on Body Weight Loss and Neurological Development of EAE

All untreated EAE-inoculated rats developed neurological signs of disease with the majority of animals suffering paralytic symptoms (Table 6.1). Administration of the dual NOS inhibitor, L-NAME, had no suppressive effect on neurological disease (Table 6.1). In contrast, treatment with i.p. vehicle significantly suppressed the development of paralytic EAE ($p < 0.05$) together with the severity of disease symptoms ($p < 0.01$) (Table 6.1). However, the profile of body weight changes in L-NAME and vehicle treated rats was similar to fluctuations recorded in undosed inoculated animals (Figure 6.1).

Treatment with either 7-NI or vehicle reduced the incidence of EAE, significantly alleviated disease severity ($p < 0.001$) and dramatically reduced paralytic symptoms ($p < 0.001$) (Table 6.1). By comparison, weight loss in 7-NI and vehicle treated animals were similar to changes observed in undosed EAE-sensitised rats (Figure 6.1).

Dosing animals with the iNOS inhibitor, AG or s.c. vehicle did not suppress the onset or development of neurological disease (Table 6.1). In addition, body weight loss experienced by rats receiving the drug was not typically in excess of values recorded for inoculated or vehicle treated animals (Figure 6.1).

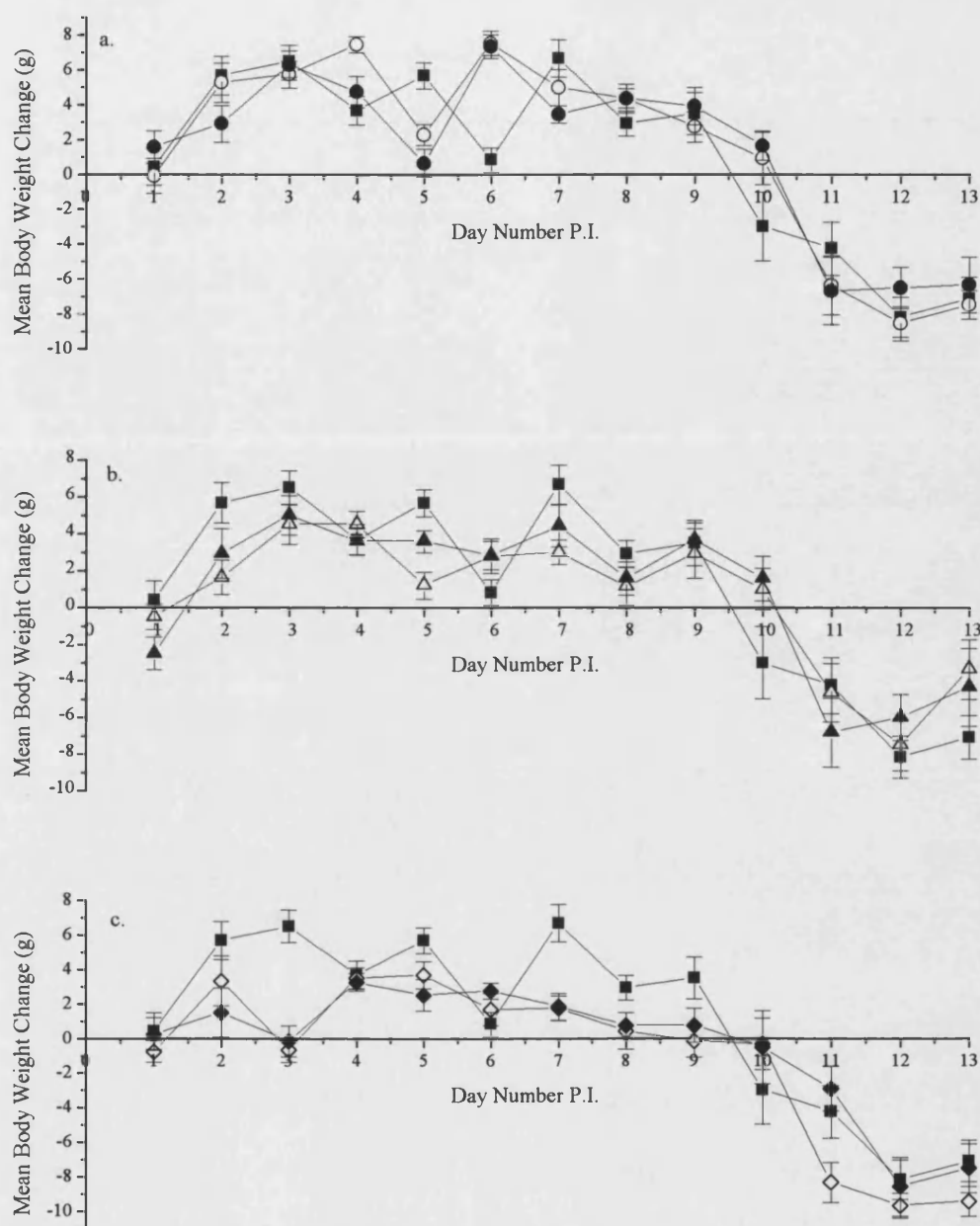


Figure 6.1 Effect of NOS inhibitors administration on body weight

Animals were weighed daily and changes in body weight were measured in g and expressed as mean body weight change \pm S.E.M.

- a. (—■—) EAE-diseased Lewis rats, n=8; (—○—) EAE-sensitised animals i.p. injected with L-NAME (30 mg/kg body weight) from days 7 to 12 P.I., n=11; (—●—) EAE-inoculated rats injected i.p. with vehicle (PBS, 1 ml/kg body weight) from days 7 to 12 P.I., n=11.
- b. (—■—) EAE-diseased animals, n=8; (—△—) EAE-inoculated Lewis rats i.p. injected with 7-NI (10 mg/kg body weight) from days 7 to 12 P.I., n=14; (—▲—) EAE-sensitised rats dosed i.p. with vehicle (peanut oil, 1 ml/kg body weight) from days 7 to 12 P.I., n=12.
- c. (—■—) EAE-diseased Lewis rats, n=8; (—◇—) EAE-sensitised rats injected s.c. with AG (200 mg/kg body weight) from day 1 to 12 P.I., n=12; (—◆—) EAE-inoculated animals injected s.c. with vehicle (PBS, 1 ml/kg body weight) from day 1 to 12 P.I., n=12.

Table 6.1 Neurological symptoms in EAE-sensitised Lewis rats following treatment with L-NAME (30 mg/kg body weight, i.p., days 7 to 12 P.I.); L-NAME vehicle (PBS, 1 ml/kg body weight, i.p., days 7 to 12 P.I.); 7-NI (10 mg/kg body weight, i.p., days 7 to 12 P.I.); 7-NI vehicle (peanut oil, 1 ml/kg body weight, i.p., days 7 to 12 P.I.); AG (200 or 400 mg/kg body weight, s.c., days 1 to 12 P.I.) and AG vehicle (PBS, 1 ml/kg body weight, s.c., days 1 to 12 P.I.).

Group	Incidence Of EAE	Mean Day Of Disease Onset \pm S.E.M.	Mean Peak Disease Severity \pm S.E.M.	Number Of Rats Paralysed/Total
EAE	16/16	12 ± 1	3 ± 0.2	11/16
L-NAME	15/15	12 ± 1	$3 \pm 0.2^{\dagger}$	8/15
Vehicle	23/24	12 ± 1	$2 \pm 0.2^{**}$	7/24*
7-NI	14/18*	12 ± 1	$1 \pm 0.2^{***}$	2/18***
Vehicle	15/18	12 ± 1	$2 \pm 0.2^{***}$	2/18***
AG (200)	12/12	11 ± 1	3 ± 0.2	8/12
AG (400)	6/6	11 ± 1	3 ± 0.3	3/6
Vehicle	18/18	12 ± 1	2 ± 0.3	6/18*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to EAE group.

$^{\dagger}p < 0.05$ compared to vehicle group.

Differences in mean day of disease onset and mean peak disease severity were analysed using the Mann Whitney U test.

Differences in the incidence of EAE and the number of rats displaying paralysis were analysed using the Chi-squared test.

6.2 Effect of NOS Inhibitor Administration on Nitrite Levels in CNS Cytosol

Nitrite levels were measured in CNS cytosol preparations from dosed and untreated EAE-diseased animals to confirm that NOS inhibitors did prevent NO formation.

Treatment of EAE-diseased animals with L-NAME decreased nitrite levels in all CNS extracts to within normal limits (cerebellum $p < 0.01$, medulla-pons $p < 0.001$) (Figure 6.2). In contrast, nitrite concentrations in samples from vehicle treated rats were comparable to levels recorded in tissues from undosed EAE-diseased animals (Figure 6.2).

7-NI administration significantly reduced CNS cytosol nitrite levels in EAE-sensitised rats (cerebellum $p < 0.001$, medulla-pons $p < 0.001$, cervical spinal cord $p < 0.01$) (Figure 6.3). Furthermore, nitrite levels in cerebellar cytosol extracts from EAE-inoculated animals treated with i.p. peanut oil vehicle were significantly decreased ($p < 0.01$). However, nitrite content in medulla-pons and cervical spinal cord tissue from vehicle dosed rats were similar to values obtained in untreated diseased animals (Figure 6.3).

EAE-sensitised Lewis rats dosed with AG had significantly diminished CNS nitrite levels (cerebellum $p < 0.05$, medulla-pons $p < 0.01$, cervical spinal cord $p < 0.05$) (Figure 6.4). Moreover, medulla-pons cytosol samples from vehicle treated animals had significantly reduced nitrite levels ($p < 0.05$). By comparison, no significant differences in nitrite content were recorded in cerebellar or cervical spinal cord tissue of EAE-diseased rats following treatment with PBS vehicle (Figure 6.4). Finally, CNS cytosol nitrite levels were not significantly different between AG and vehicle treated rats (Figure 6.4).

6.3 Effect of NOS Inhibitor Administration on ROS Levels in CNS Cytosol

The superoxide scavenging capacity of NO has previously been documented (Guitierrez *et al.* 1996), therefore decreasing NO synthesis may alter the production of ROS. Consequently superoxide and hydrogen peroxide levels were measured in CNS

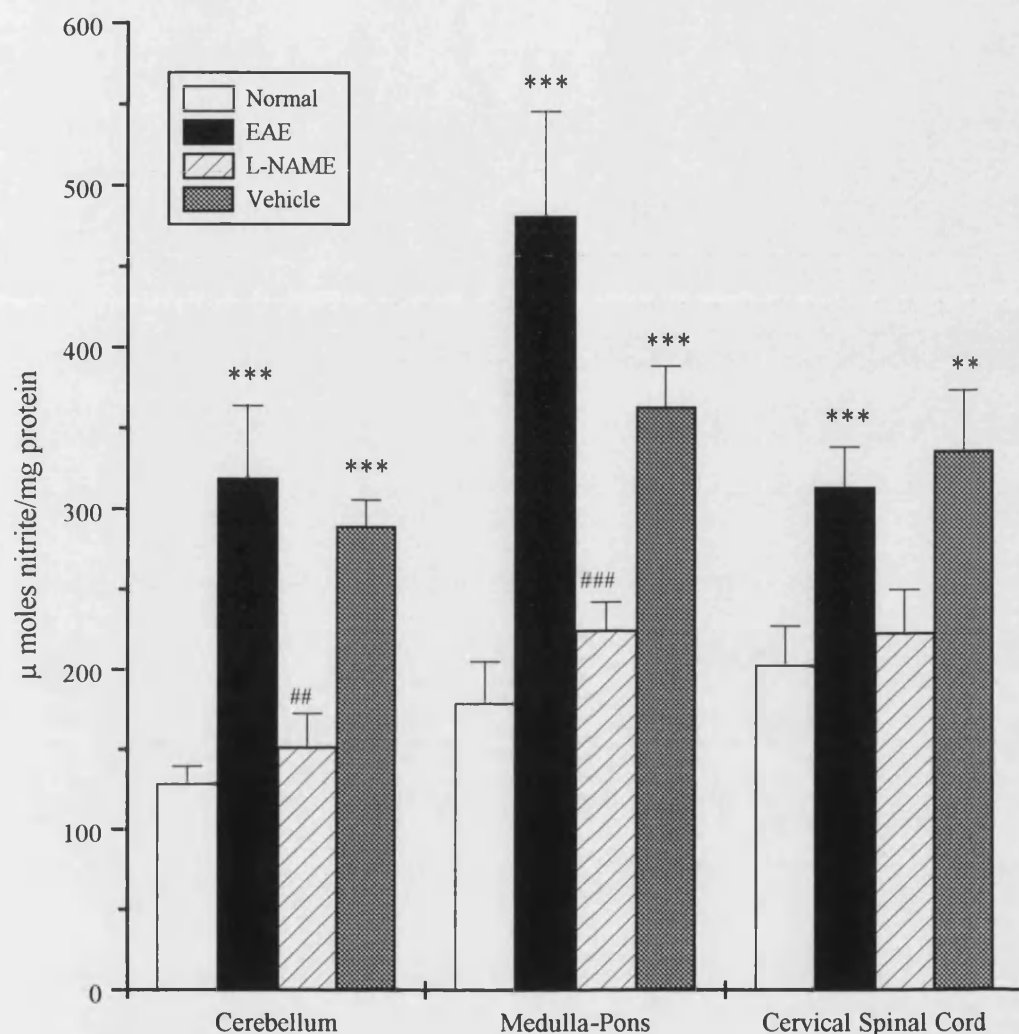


Figure 6.2 Effect of L-NAME administration on nitrite levels in CNS cytosol
 CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, $n = 12$; EAE-diseased Lewis rats, $n = 12$; EAE-sensitised Lewis rats i.p. injected with L-NAME (30mg/kg body weight) days 7 to 12 P.I., $n = 10$; EAE-inoculated Lewis rats dosed i.p. with vehicle (PBS, 1ml/kg body weight) days 7 to 12 P.I., $n = 13$. ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. ## $p < 0.01$, ### $p < 0.001$ Mann Whitney U test compared to EAE animals.

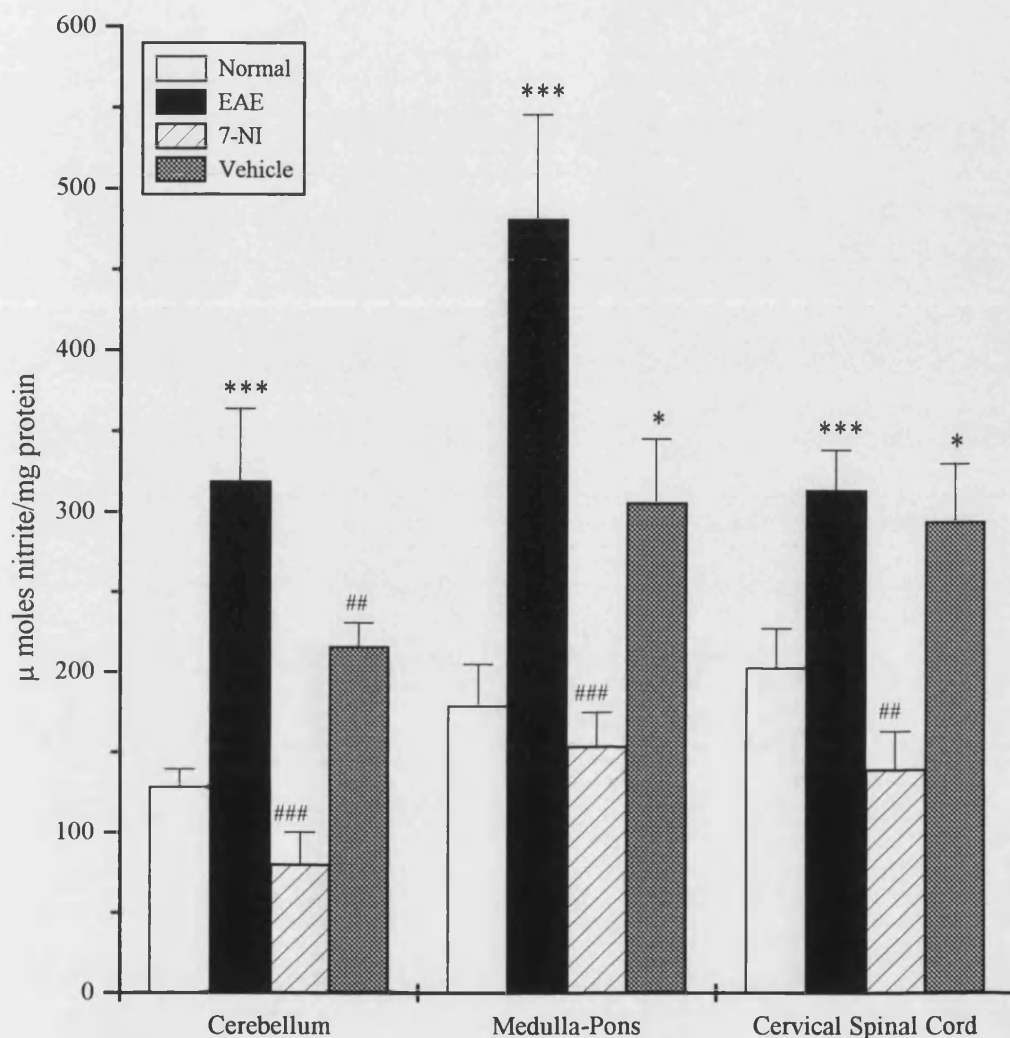


Figure 6.3 Effect of 7-NI administration on nitrite levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, $n = 12$; EAE-diseased Lewis rats, $n = 12$; EAE-sensitised Lewis rats i.p. injected with 7-NI (10 mg/kg body weight) days 7 to 12 P.I., $n = 12$; EAE-inoculated Lewis rats dosed i.p. with vehicle (peanut oil, 1ml/kg body weight) days 7 to 12 P.I., $n = 12$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. ## $p < 0.01$, ### $p < 0.001$ Mann Whitney U test compared to EAE animals.

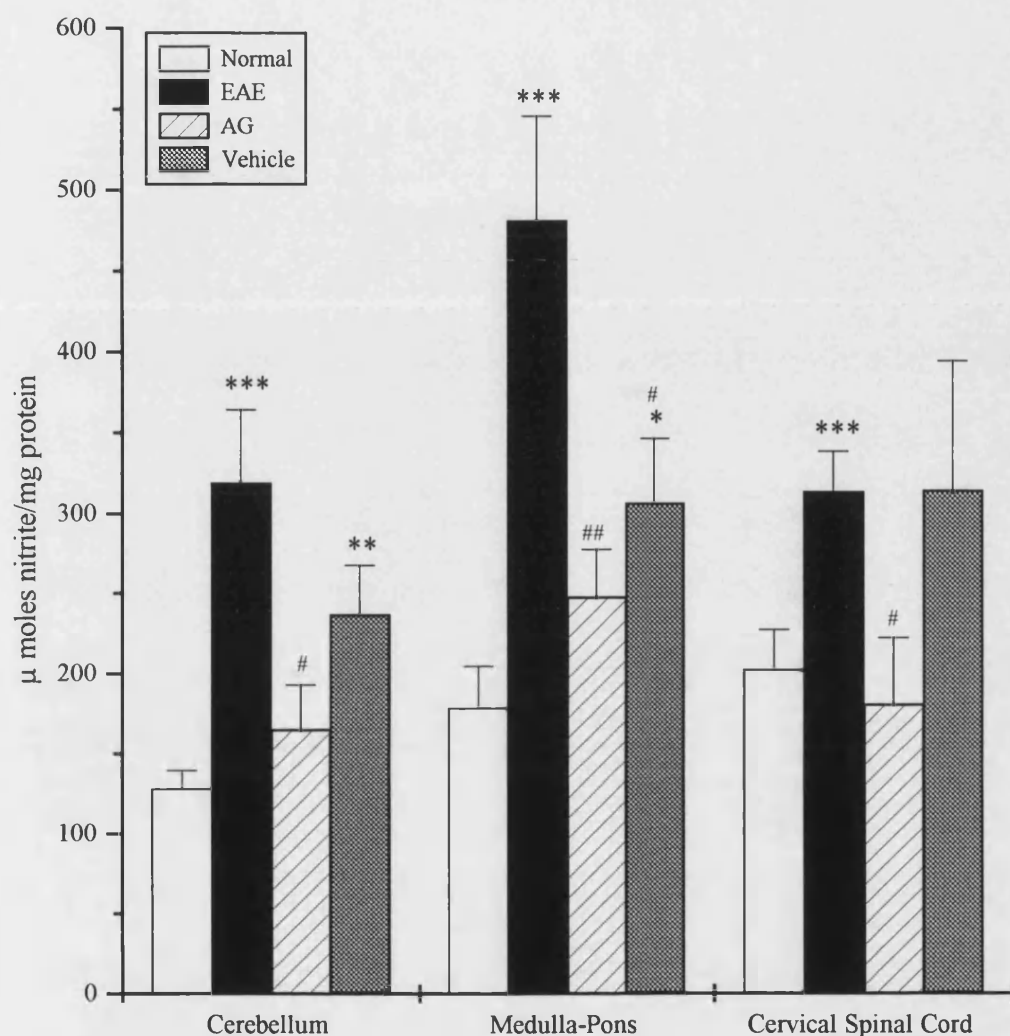


Figure 6.4 Effect of AG administration on nitrite levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, $n = 12$; EAE-diseased Lewis rats, $n = 12$; EAE-sensitised Lewis rats s.c. injected with AG (200mg/kg body weight) days 1 to 12 P.I., $n = 11$; EAE-inoculated Lewis rats dosed s.c. with vehicle (PBS, 1ml/kg body weight) days 1 to 12 P.I., $n = 11$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. # $p < 0.05$, ## $p < 0.01$ Mann Whitney U test compared to EAE animals.

cytosol samples from drug treated animals to determine if NOS inhibitors effect ROS generation in EAE-diseased Lewis rats..

6.3.1 Effect of L-NAME Administration on ROS Levels in CNS Cytosol

Superoxide levels in the cerebellum and cervical spinal cord of EAE-diseased animals were unaffected by the administration of L-NAME (Figure 6.5a). In contrast, the superoxide content of medulla-pons cytosol fraction was significantly reduced following L-NAME treatment ($p<0.05$) (Figure 6.5a). Similarly, administration of i.p. PBS vehicle to EAE-sensitised rats did not significantly alter cerebellar or cervical spinal cord cytosol superoxide levels but significantly decreased superoxide content of medulla-pons tissue ($p<0.05$) (Figure 6.5a). No differences existed between the superoxide content of L-NAME or vehicle treated animals CNS cytosol (Figure 6.5a).

Administration of L-NAME significantly reduced CNS cytosol hydrogen peroxide levels in EAE-inoculated animals (cerebellum $p<0.05$, medulla-pons $p<0.01$, cervical spinal cord $p<0.05$) (Figure 6.5b). In addition hydrogen peroxide levels in CNS cytosol extracts from vehicle treated animals were reduced (Figure 6.5b).

6.3.2 Effect of 7-NI Administration on ROS Levels in CNS Cytosol

Treatment of EAE-diseased Lewis rats with 7-NI had no effect on cerebellar cytosol superoxide content but significantly decreased the levels in medulla-pons and cervical spinal cord tissues ($p<0.01$) (Figure 6.6a). Furthermore, vehicle administration had comparable effects on the superoxide content of CNS cytosol from EAE-inoculated animals (Figure 6.6a).

Hydrogen peroxide levels in the cerebella of EAE-sensitised animals were significantly reduced by 7-NI administration ($p<0.05$) (Figure 6.6b). By comparison, medulla-pons and cervical spinal cord cytosol hydrogen peroxide content was not significantly altered following treatment with 7-NI (Figure 6.6b). Administration of i.p. peanut oil vehicle significantly decreased hydrogen peroxide levels in cerebella and medulla-pons tissue ($p<0.05$) but not in cervical spinal cord cytosol (Figure 6.6b).

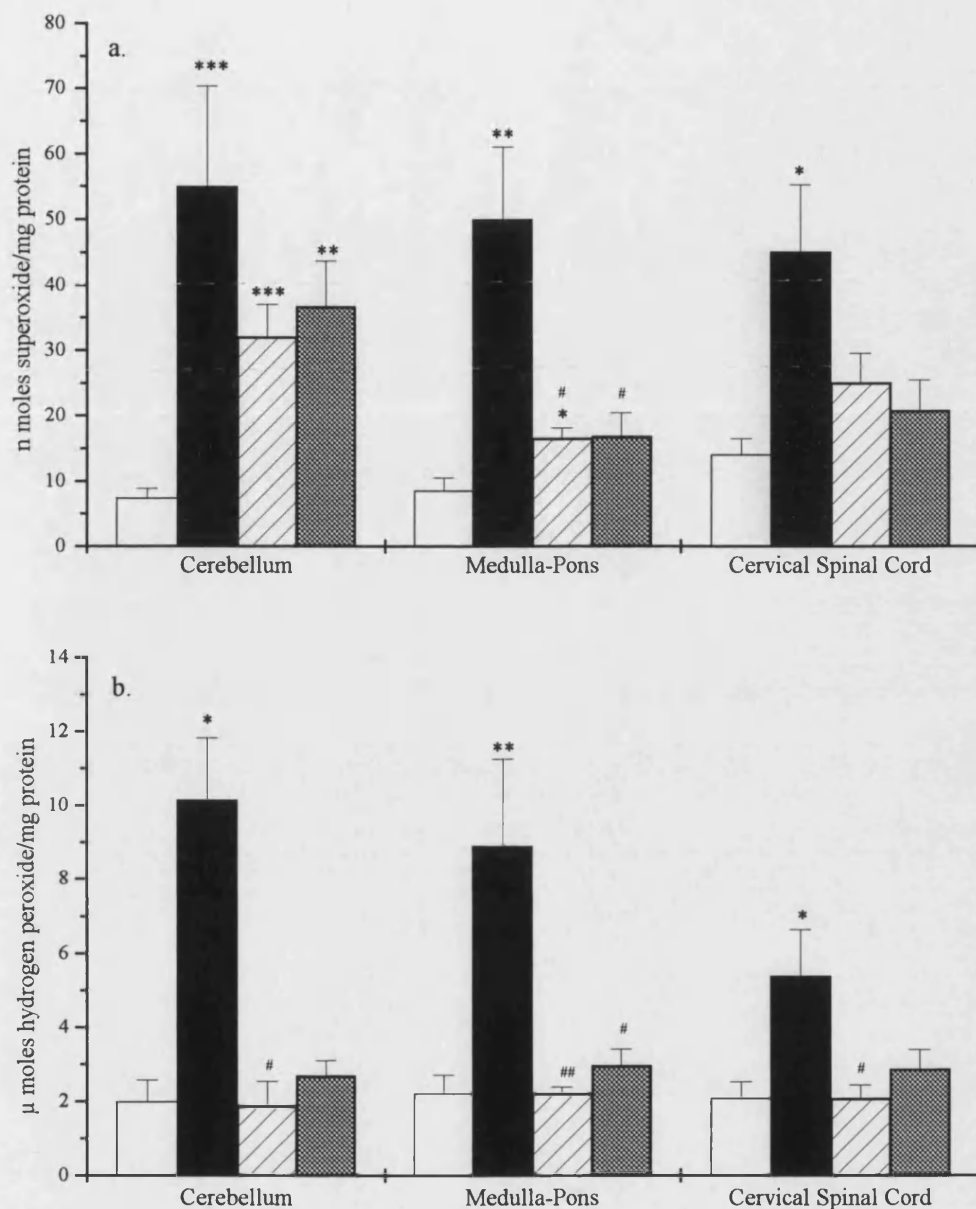


Figure 6.5 Effect of L-NAME administration on ROS levels in CNS cytosol
CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;

▨ EAE-sensitised Lewis rats i.p. injected with L-NAME (30mg/kg body weight) on days 7 to 12 P.I., n = 7; ▤ EAE-inoculated Lewis rats dosed i.p. with vehicle (PBS, 1ml/kg body weight) from day 7 to 12 P.I., n = 7.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. # $p < 0.05$, ## $p < 0.01$ Mann Whitney U test compared to EAE animals.

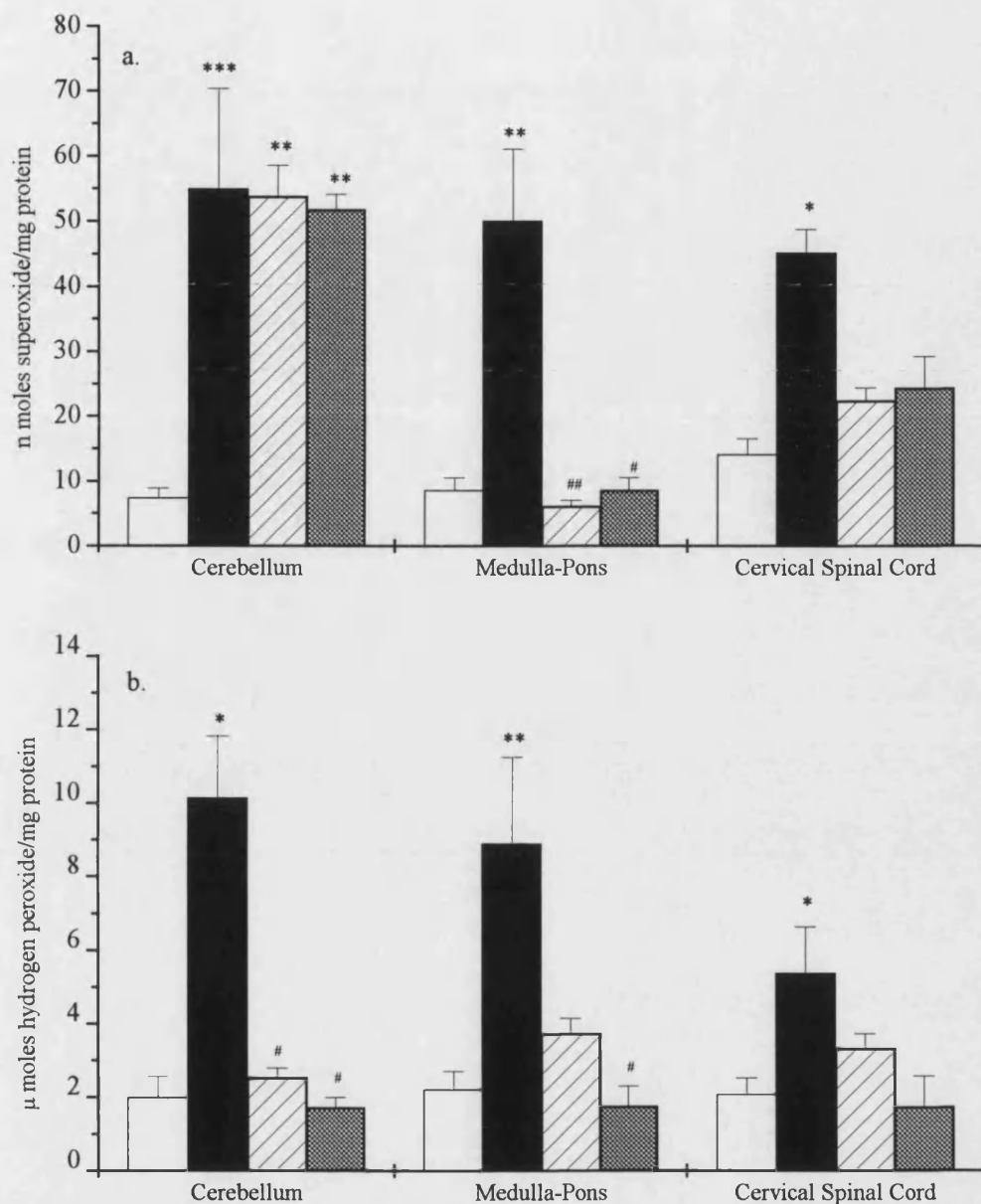


Figure 6.6 Effect of 7-NI administration on ROS levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;
 ▨ EAE-sensitised Lewis rats i.p. injected with 7-NI (10 mg/kg body weight) from days 7 to 12 P.I., n = 6; ▩ EAE-inoculated Lewis rats i.p. injected with vehicle (PBS, 1ml/kg body weight) from days 7 to 12 P.I., n = 6. *p<0.05, **p<0.01, ***p<0.001 Mann Whitney U test compared to normal animals.
 #p<0.05, ##p<0.01 Mann Whitney U test compared to EAE animals.

6.3.3 Effect of AG Administration on ROS Levels in CNS Cytosol

EAE-inoculated Lewis rats treated with AG showed significantly reduced superoxide levels in cytosol from medulla-pons tissue ($p < 0.05$) (Figure 6.7a). However, superoxide content in cerebellar and cervical spinal cord tissue were unaffected by AG administration (Figure 6.7a). Moreover CNS cytosol superoxide levels were unaffected by s.c. vehicle treatment of EAE-sensitised rats (Figure 6.7a).

AG administration significantly reduced CNS cytosol levels of hydrogen peroxide in EAE-diseased animals ($p < 0.05$) (Figure 6.7b). Vehicle treatment did not alter medulla-pons or cervical spinal cord hydrogen peroxide levels but did significantly decrease the content of cerebella cytosol preparations ($p < 0.05$) (Figure 6.7b).

6.4 Effect of NOS Inhibitors On Cellular Infiltration

The ability of NOS inhibitors to alter inflammatory cell infiltration during EAE were assessed by determining the number of perivascular infiltrates in cervical spinal cord sections (Figure 6.8).

Administration of L-NAME or vehicle did not effect cell migration during EAE (Table 6.2). By comparison, 7-NI and peanut oil vehicle treatment significantly reduced cellular infiltration in EAE ($p < 0.01$) (Table 6.2). Similarly, dosing with AG or s.c. vehicle decreased inflammatory cell infiltration during EAE ($p < 0.01$) (Table 6.2). However, no differences existed between the number of perivascular infiltrates from drug or vehicle treated animals.

6.5 Effect of NOS Inhibitors on Serum Corticosterone Levels

Corticosterone levels were measured in sera samples from EAE-sensitised animals treated with NOS inhibitor or vehicle. EAE was associated with an increase in sera levels of corticosterone (Table 6.3). Administration of NOS inhibitors decreased sera corticosterone content. Additionally, vehicle treatment reduced sera corticosterone levels (Table 6.3). However, the corticosterone content of L-NAME and AG vehicle treated sera was slightly higher than in the respective drug treated samples (Table 6.3).

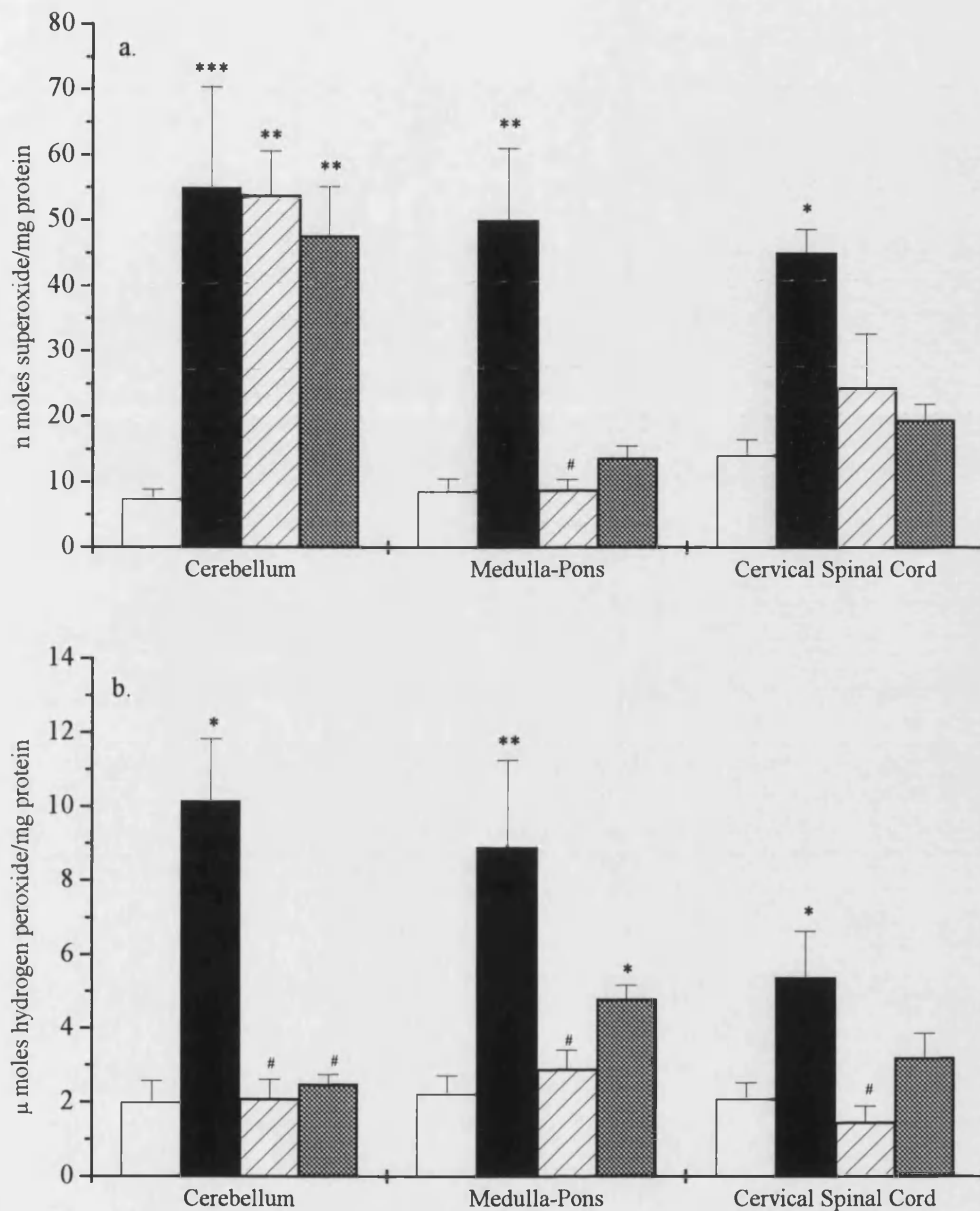


Figure 6.7 Effect of AG administration on ROS levels in CNS cytosol

CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;
 ▨ EAE-sensitised Lewis rats s.c. injected with AG (200mg/kg body weight) from day 1 to 12 P.I., n = 6; ▩ EAE-inoculated Lewis rats s.c. dosed with vehicle (PBS, 1ml/kg body weight) from day 1 to 12 P.I., n = 6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals.

$p < 0.05$ Mann Whitney U test compared to EAE animals.

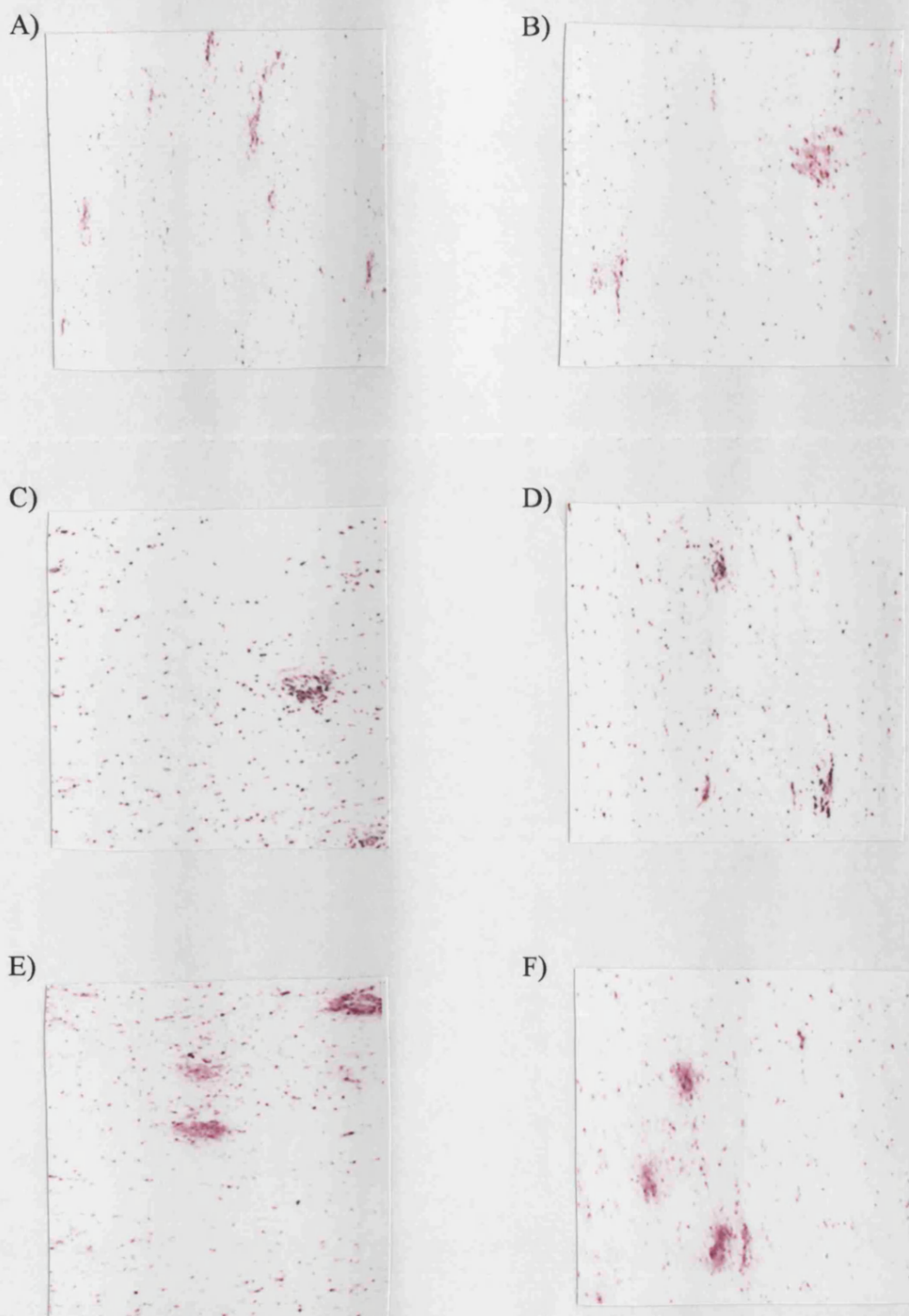


Figure 6.8 Effect of NOS inhibitors on cellular infiltration during EAE

Cellular infiltration in haematoxylin and eosin stained cervical spinal cord samples from EAE-sensitised animals following administration of A) L-NAME (30 mg/kg body weight, i.p., days 7 to 12 P.I.), B) PBS vehicle (1 ml/kg body weight, i.p., days 7 to 12 P.I.), C) 7-NI (10 mg/kg body weight, i.p. days 7 to 12 P.I.), D) Peanut oil vehicle (1 ml/kg body weight, i.p., days 7 to 12 P.I.), E) AG (200 mg/kg body weight, s.c., days 1 to 12 P.I.) and F) PBS vehicle (1 ml/kg body weight, s.c., days 1 to 12 P.I.). Magnification x40.

Table 6.2 Perivascular infiltrates in cervical spinal cord samples from EAE-sensitised Lewis rats following treatment with L-NAME (30 mg/kg body weight, i.p., days 7 to 12 P.I.), 7-NI (10 mg/kg body weight, i.p., days 7 to 12 P.I.) and AG (200mg/kg body weight, s.c., days 1 to 12 P.I.) or the appropriate vehicle.

Group	Mean Number of Lesions ^a /10 mm Section (\pm S.E.M.)
EAE	53 \pm 4
L-NAME	52 \pm 3
Vehicle	45 \pm 3
7-NI	28 \pm 4***
Vehicle	37 \pm 4 ***
AG	33 \pm 1***
Vehicle	37 \pm 2**

^a Sections were cut at one standard depth, stained with haematoxylin and eosin and the total number of lesions per section was determined using light microscopy (x40). Values are means of 18 sections from 3 animals \pm S.E.M. **p<0.01, ***p<0.001 Mann Whitney U test compared to EAE group.

Table 6.3 Serum corticosterone levels from normal, EAE-diseased and EAE-sensitised Lewis rats following treatment with L-NAME (30 mg/kg body weight, i.p., days 7 to 12 P.I.), 7-NI (10 mg/kg body weight, i.p., days 7 to 12 P.I.) and AG (200mg/kg body weight, s.c., days 1 to 12 P.I.) or the appropriate vehicle.

Group	Corticosterone (ng/ml) ^a (± S.E.M.)
Normal	52 ± 1
EAE	107 ± 23
L-NAME	56 ± 6
Vehicle	61 ± 2
7-NI	59 ± 5
Vehicle	59 ± 5
AG	57 ± 4
Vehicle	68 ± 4

^a Corticosterone levels were measured using a commercially available RIA kit. Values are means of 4 animals ± S.E.M.

6. DISCUSSION

Several studies have provided strong evidence of a role for NO in the development of EAE and the pathogenesis of MS. Investigations by Bö *et al.* (1994) and Koprowski *et al.* (1993) have demonstrated elevated levels of NOS mRNA in the CNS of MS patients and EAE-diseased animals respectively. Interestingly, the observed iNOS mRNA levels detected during EAE correlated with disease severity. Complementary work by other groups has also shown that levels of NOS enzymes and related metabolites increase during EAE (Hooper *et al.* 1995, Misko *et al.* 1995, Okuda *et al.* 1995, Van Dam *et al.* 1995, Hewett *et al.* 1996). Furthermore, a role for NO in the occurrence of EAE and MS is implicated through indirect evidence demonstrating NO-mediated oligodendrocyte cytotoxicity, neuronal degeneration and demyelination (Merrill *et al.* 1993, Skaper *et al.* 1995). A more direct approach to establishing the importance of NO in the aetiology of EAE has been undertaken in the present study by pharmacological manipulation of the disease with NOS inhibitors.

6.1 NOS Inhibitors in EAE

Previous work has shown a discrepancy in the effects of NOS inhibitors on the neurological course of EAE. For example, dosing with the c and iNOS inhibitor, L-NAME, either exacerbated or had no effect on disease symptoms (Zielasek *et al.* 1995a, Ruuls *et al.* 1996). Treatment of EAE-sensitised animals with a related non-selective NOS inhibitor, L-nitroarginine, either suppressed or enhanced neurological signs (Ovadia *et al.* 1994, Ruuls *et al.* 1994). In addition, administration of the selective iNOS inhibitor, AG, to EAE-inoculated rats was shown to abrogate or aggravate disease progression (Cross *et al.* 1994, Zielasek *et al.* 1995a, Zhao *et al.* 1996).

Although the results of the study examining the effect of AG on neurological EAE do not corroborate previous findings (Zielasek *et al.* 1995a, Zhao *et al.* 1996), effects of L-NAME on disease development are in agreement with the observations of Ruuls *et al.* (1996). However, the effects of 7-NI administration on neurological disease symptoms

have not been previously assessed. The variability of identical drugs on the development and severity of neurological EAE may be due to several factors including species difference, EAE-induction methods and dosing regimen employed in each study. Indeed the lack of effect of AG in this study compared to the abrogation and aggravation of EAE demonstrated by the studies of Zielasek *et al.* (1995a) and Zhao *et al.* (1996) may result from differences in the route and time course of drug administration.

Low dose administration of the selective iNOS inhibitor, AG (Griffiths *et al.* 1993) to EAE-inoculated rats did not modify disease development. Furthermore increasing the dose of AG failed to alter the appearance and course of EAE. Similarly, treatment of EAE-sensitised Lewis rats with the c and iNOS, L-NAME (Rees *et al.* 1990b) did not significantly affect the incidence or severity of disease. Interestingly, recent work by Ruuls *et al.* demonstrates that EAE is aggravated by L-NAME administration. The present study reconfirms these observations as disease signs in L-NAME treated rats were significantly exacerbated in comparison to rats administered i.p. vehicle. In contrast a reduction in the incidence and severity of EAE was apparent when animals were administered 7-NI, a selective inhibitor of bNOS (Moore *et al.* 1993). However the significance of the results becomes complicated following the unexpected modulatory effects of vehicle administration on disease symptoms. Support for the ability of vehicle treatment to modify the course of EAE is provided by the earlier study of Leonard *et al.* (1990) and more recent work described by Hewson *et al.* (1995). Clearly when determining the effects of NOS inhibitors on the development of EAE the consequences of vehicle administration should be adequately assessed.

Disease suppression by vehicle treatment alone may be explained, in part, by the production and well-established modulatory actions of endogenous glucocorticoids on the progression of EAE (Levine *et al.* 1962, Bolton & Flower 1989, MacPhee *et al.* 1989). Circulating corticosteroid levels increase during stress (Weicker & Werle 1991) such as undoubtedly occurs following repeated i.p. or long term s.c. injections of vehicle. Therefore, naturally occurring glucocorticoids may be abnormally increased during the induction phase of EAE and thus influence the appearance of disease symptoms.

Preliminary data from the present study demonstrates a slight increase in serum corticosterone levels following vehicle treatment. However, corticosterone in vehicle rat serum was markedly reduced compared to undosed sensitised animals. This may be accounted for, in part, by experimental problems with the corticosterone detection system used as the radioimmunoassay employed was designed for measuring corticosterone in plasma and not serum samples. Furthermore, levels of corticosterone measured in diluted samples were at the detection limit of the assay which may lead to inaccurate measurements. In addition the low level of corticosterone present in the sera of vehicle treated rats may be due to an alteration in the steroid profile of the animal. Therefore, the peak steroid levels may have occurred prior to sampling. To determine the validity of this hypothesis corticosteroid levels would have to be determined at various timepoints throughout vehicle treatment.

6.2 NOS Inhibitors and NO Generation

Although other workers have utilised NOS inhibitors to modify the course of EAE the studies have not assessed the ability of the compounds to prevent NO formation in CNS tissues. Indeed NOS inhibitors are readily transported into the CNS (Bogle *et al.* 1992b, Iadecola *et al.* 1994) where they prevent NO synthesis through effects on NOS activity. The present investigation has quantitatively determined the *in vivo* pharmacological effect of the NOS inhibitors, L-NAME, 7-NI and AG, and vehicle treatment on NO generation by CNS tissues in EAE-diseased Lewis rats. Administration of NOS inhibitors to EAE-sensitised animals caused a decrease in CNS cytosol nitrite levels indicating the functional activity of the compounds to prevent NO production.

6.2.1 L-NAME

L-NAME is a well characterised L-arginine analogue which inhibits the activity of both c and iNOS (Rees *et al.* 1990, McCall *et al.* 1991a). However, L-NAME is a weak inhibitor of NOS and requires conversion to N^G-nitro-L-arginine (L-NA) to become fully active (Pfeiffer *et al.* 1996). Activated L-NAME functions as a competitive inhibitor of

the substrate binding site of NOS to prevent NO formation (Griffith & Kilbourn 1996). In addition L-NA induces a time dependent NOS enzyme inactivation (Klatt *et al.* 1994) through conformational changes at the active site (Reif & McCreedy 1995).

The current study has shown that L-NAME administration reduced nitrite levels in the CNS of EAE-disease animals to within normal limits. The observation is surprising due to the non-specificity of NOS inhibition by L-NAME. Indeed L-NAME may be expected to decrease nitrite levels to below normal values due to its ability to inhibit iNOS as well as basal cNOS activity. However, there may be several explanations to account for the limited effect of drug administration on nitrite generation. Although L-NAME inhibits the activity of both c and iNOS enzymes a slight selectivity for the c isoform has been demonstrated (Furfin *et al.* 1993). Therefore in EAE drug treatment may only prevent c and not iNOS activity and so account for enhanced nitrite levels. L-NAME can induce the expression of iNOS (Miller *et al.* 1996a). Hence any inhibitory effects of the drug on cNOS generated nitrite during EAE would be reversed by the increased nitrite production by iNOS. Nitrite levels in EAE-inoculated animals may remain higher than expected subsequent to L-NAME administration due to the ability of the drug to act as an alternative substrate for NOS (Komori *et al.* 1994). However, further studies are required to determine the precise mechanisms responsible for this elevation.

6.2.2 7-NI

7-NI is a potent and selective inhibitor of bNOS (Moore *et al.* 1993) which has previously been utilised in the treatment of CNS disorders (Schulz *et al.* 1995). The activity of bNOS is inhibited by the competitive binding of 7-NI to the substrate site on the enzyme (Babbedge *et al.* 1993). In addition the drug has been identified as a competitive inhibitor of the tetrahydrobiopterin site (Mayer *et al.* 1994). There is evidence to suggest that 7-NI binds to the haem prosthetic site of NOS which prevents access of both L-arginine and tetrahydrobiopterin (Mayer *et al.* 1994).

In the current study dosing EAE-sensitised Lewis rats with 7-NI produced a decrease in CNS tissue nitrite levels to below values recorded in normal animals. Clearly

drug administration would be expected to prevent basal NO production from bNOS and so reduce CNS nitrite levels to below normal limits. In addition recent investigations have demonstrated that 7-NI also inhibits eNOS activity *in vivo* (Zagvazdin *et al.* 1996) contributing to the ability of the drug to inhibit basal NOS and decrease CNS nitrite levels below normal values. EAE is an immunologically-mediated disorder and the majority of CNS nitrite may be generated by the iNOS isoform. However, iNOS is not inhibited by 7-NI. Therefore the results suggest that the majority of nitrite formed in the CNS of diseased animals is generated via cNOS isoforms. Recent *in vitro* studies have shown that 7-NI has a slight inhibitory effect on iNOS (Wolff & Grisbin 1994). Hence inhibition of all 3 isoforms of NOS may be responsible for the observed effects in EAE.

6.2.3 AG

AG has been identified as a selective inhibitor of iNOS (Corbett *et al.* 1992b, Griffiths *et al.* 1993, Joly *et al.* 1993, Misko *et al.* 1993). However, more recent studies have identified that cNOS isoforms are also inhibited by the drug (Laszlo *et al.* 1995, Lopez-Belmonte & Whittle 1995). Inhibition of NOS by AG is mechanism based with the NO forming activity of the enzyme being prevented (Wolff & Lubeskie 1995).

EAE-inoculated Lewis rats were administered AG to determine the effects of selectively inhibiting iNOS in the model. Moreover, a reduction in CNS nitrite levels was detected following drug treatment. However, in view of recent investigations it cannot be concluded that the decrease in nitrite content is due solely to an action of AG on iNOS.

6.3 Other Effects of NOS Inhibitors

In addition to effects on NO formation, NOS inhibitors may exert other actions in the CNS to modify the course of EAE.

6.3.1 ROS

ROS have been identified as potential cytotoxic molecules in EAE (Bowern *et al.* 1984). Furthermore the NOS inhibitors, L-NAME and AG have previously been shown

to affect ROS generation. Whereas L-NAME administration prevents ROS formation, treatment with AG enhances ROS production (Heinzel *et al.* 1992, Ou & Wolff 1993). By comparison 7-NI has not been shown to affect ROS generation. In the present study ROS levels in the medulla-pons and cervical spinal cord were reduced following NOS inhibitor administration. However, the decrease was not always associated with a reduction in disease severity. Furthermore, similar effects were detected subsequent to the respective vehicle treatment.

6.3.2 Cell Infiltration

NO has previously been identified as an anti-adhesion molecule, but the underlying mechanisms are unknown (Gaboury *et al.* 1993). Therefore dosing with NOS inhibitors may increase immunocompetent cell infiltration during EAE and subsequently exacerbate disease development. However, in the present study drug administration reduced the number of perivascular isolates in the spinal cord of EAE-inoculated rats. Vehicle treatment also decreased cellular infiltration. These findings indicate that NOS inhibitors may not specifically alter cell migration in EAE.

6.3.3 Non-Specific Effect of AG

Although L-NAME and 7-NI exert their effects through the inhibition of NOS and NADPH oxidation, AG is known to have a broad profile of pharmacological and biochemical actions. Therefore AG may affect the neurological development of EAE through actions on diamine oxidase, aldose reductase, polyamine metabolism and macrophage uptake of low density lipoproteins (Kumari *et al.* 1991, Ohri *et al.* 1992, Picard *et al.* 1992). However, as none of these factors has an established role in EAE aetiology it is unlikely that AG alters EAE through effects on these components.

CHAPTER 7

NMDA-RECEPTOR

ANTAGONISTS

7. RESULTS

NMDA receptor activation in the CNS is involved in both neurodegeneration (Foster *et al.* 1990) and ischaemic injury (Meldrum *et al.* 1987). One of the mechanisms through which NMDA receptor activation mediates CNS damage is via the production of NO and ROS. Nitrite and ROS levels were elevated in the CNS of EAE-diseased animals suggesting that NMDA receptor activation may be involved in the disease pathogenesis. In order to further investigate this hypothesis the effects of administration of an NMDA receptor antagonist, MK-801 (Wong *et al.* 1986), on both EAE development and other disease parameters were assessed.

7.1 Effect of MK-801 Administration on Body Weight Loss and Neurological Development of EAE

MK-801 at a dose of 0.3 mg/kg body weight was administered i.p. in PBS vehicle to EAE-sensitised rats once daily from day 7 to 12 P.I. Both dosed and undosed animals were examined daily for body weight changes (Figure 7.1) and neurological disease signs (Table 7.1). All untreated EAE-sensitised rats developed neurological disease with the majority of inoculated animals suffering paralytic symptoms (Table 7.1). However, administration of MK-801 not only decreased the incidence of EAE ($p<0.05$), but also significantly alleviated disease severity ($p<0.001$) and dramatically reduced paralytic symptoms ($p<0.001$) (Table 7.1). In addition, treatment with i.p. vehicle also curtailed neurological disease severity ($p<0.01$) and significantly suppressed the development of paralytic EAE ($p<0.05$). Although MK-801 and vehicle administration abrogated disease severity the extent to which this occurred was significantly different between the two treatments ($p<0.001$) (Table 7.1). The profile of body weight changes in MK-801 and vehicle-treated rats were similar to fluctuations recorded in undosed inoculated animals (Figure 7.1).

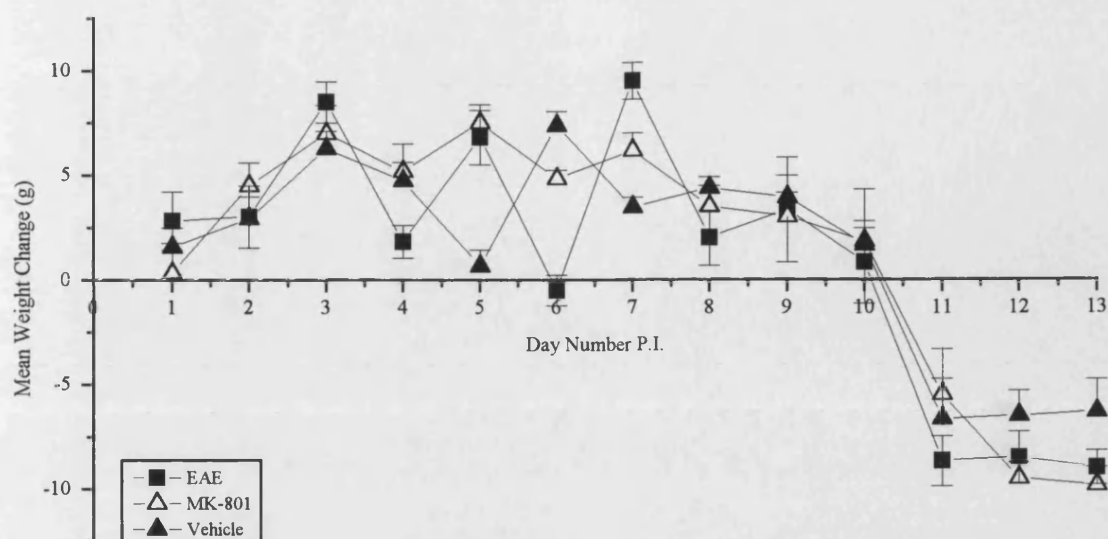


Figure 7.1 Effect of MK-801 administration on body weight

Animals were assessed daily for changes in body weight which were measured in g and expressed as mean body weight change \pm S.E.M. (—■—) EAE-inoculated Lewis rats, $n = 6$; (—△—) EAE-sensitised Lewis rats administered MK-801 (0.3 mg/kg body weight), i.p., once daily from day 7 to 12 P.I., $n = 6$; (—▲—) EAE-inoculated Lewis rats i.p. injected with vehicle (PBS, 1 ml/kg body weight) once daily from day 7 to 12 P.I., $n = 11$.

Table 7.1 Neurological symptoms in EAE-sensitised Lewis rats after treatment with MK-801 (0.3 mg/kg body weight, i.p., days 7 to 12 P.I.) or PBS vehicle (1 ml/kg body weight, i.p., days 7 to 12 P.I.).

Group	Incidence Of EAE	Mean Day Of Disease Onset \pm S.E.M.	Mean Peak Disease Severity \pm S.E.M.	Number Of Rats Paralysed/Total
EAE	16/16	12 \pm 1	3 \pm 0.2	11/16
MK-801	12/18*	13 \pm 1	1 \pm 0.3****†	1/18***
Vehicle	23/24	12 \pm 1	2 \pm 0.2**	7/24*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to EAE group.

†† $p < 0.001$ compared to vehicle group.

Differences in mean day of onset and mean peak disease severity were analysed using the Mann Whitney U test. Differences in incidence of disease and number of animals displaying signs of paralysis were analysed using the Chi-squared test.

7.2 Effect of MK-801 Administration on Nitrite Levels in CNS Cytosol

CNS cytosol preparations from MK-801 and vehicle treated animals were assayed for the presence of nitrite to determine whether the drug exerts its effects on EAE by decreasing NO production (Figure 7.2).

Treatment of EAE-diseased Lewis rats with MK-801 decreased nitrite levels in cerebellar cytosol fractions to within normal limits (MK-801 173 ± 37 , $n=7$; normal 128 ± 11 μ moles nitrite/mg protein \pm S.E.M., $n=10$) (Figure 7.2). However, MK-801 administration did not significantly alter nitrite concentrations in the other CNS areas examined (Figure 7.2). Furthermore, the nitrite content of CNS cytosol samples from vehicle treated rats were similar to levels recorded in tissues from EAE-diseased animals (Figure 7.2).

7.3 Effect of MK-801 Administration on NOS Protein Expression

In addition to effects on NO production, the ability of MK-801 to alter NOS protein expression in the CNS was examined. NOS protein expression was determined in CNS cytosol fractions from EAE-inoculated rats treated with MK-801 or vehicle using SDS-PAGE and Western blotting techniques.

8.3.1 Effect of MK-801 Administration on NOS Protein Expression in the Cerebellum

Cerebellar cytosol samples from all groups analysed contained protein bands at approximately 140 kDa, 155 kDa and 135 kDa following immunoblotting with anti-eNOS, anti-bNOS and anti-iNOS antibodies respectively. In addition the immunoreactive bands observed co-migrated with the positive control lysate for each antibody thereby indicating that the bands present correspond to each isoform of NOS (Figure 7.3).

Expression of eNOS was enhanced in the cerebellum tissue of EAE-diseased animals (34 % above normal) (Figure 7.3A). However, following MK-801 administration eNOS protein levels had fallen to below normal values (15 % below normal) (Figure

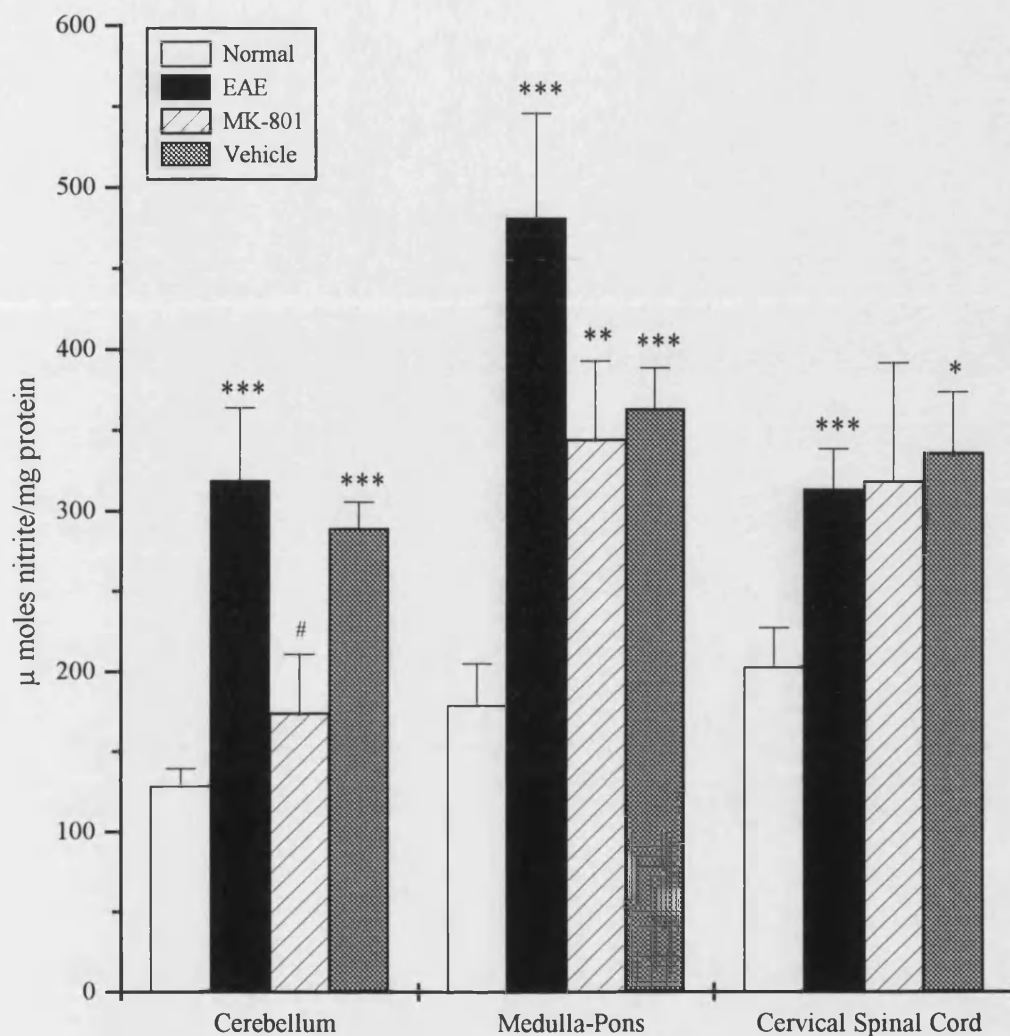


Figure 7.2 Effect of MK-801 administration on nitrite levels in CNS cytosol
 CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. Normal Lewis rats, n = 12; EAE-diseased Lewis rats, n = 12; EAE-sensitised Lewis rats i.p. injected with MK-801 (0.3mg/kg body weight) days 7 to 12 P.I., n = 7; EAE-inoculated Lewis rats dosed i.p. with vehicle (PBS, 1ml/kg body weight) days 7 to 12 P.I., n = 13. **p<0.01, ***p<0.001 Mann Whitney U test compared to normal animals. #p<0.05 Mann Whitney U test compared to EAE animals.

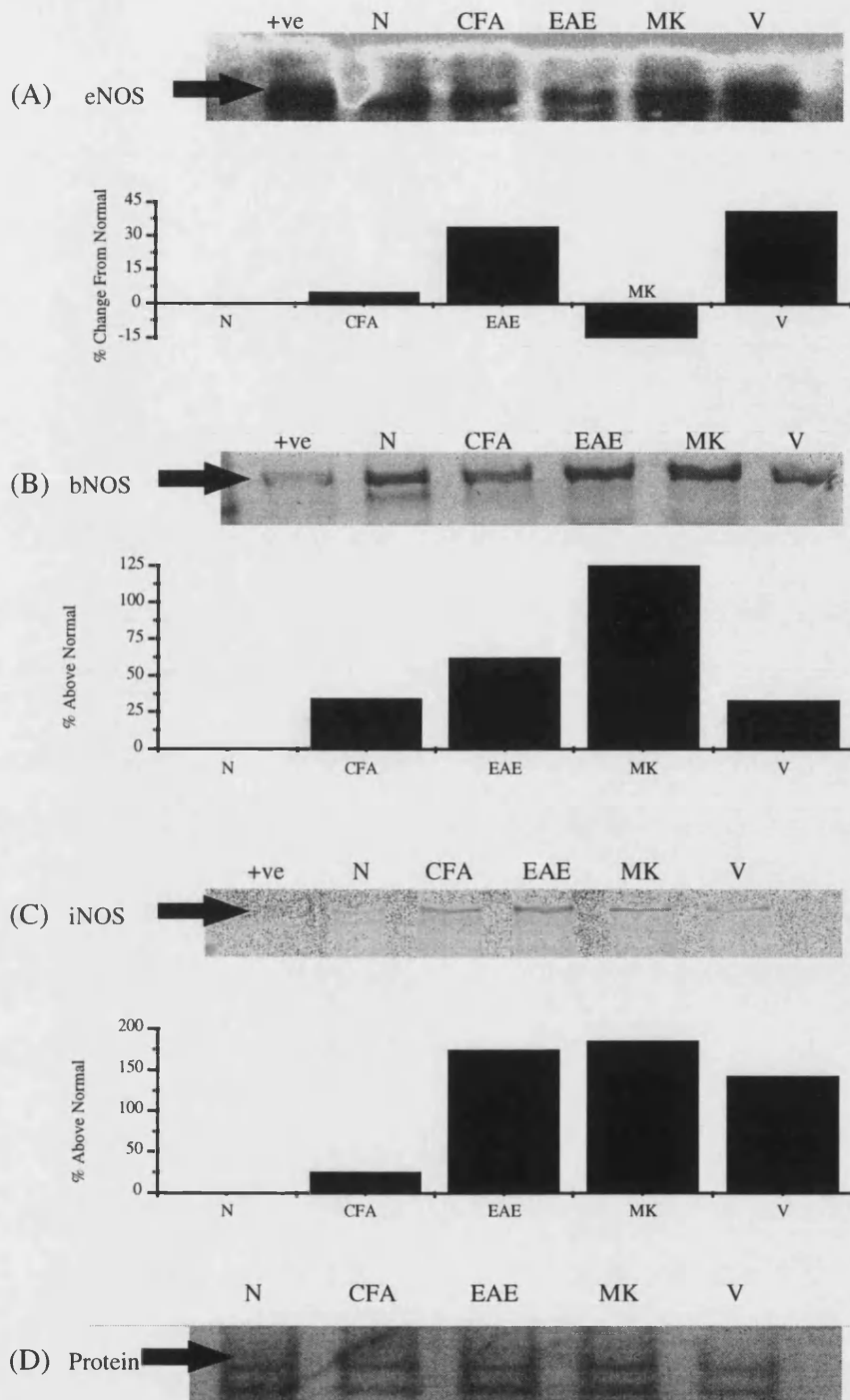


Figure 7.3 Effect of MK-801 administration on NOS protein expression

CNS cytosol was pooled from the cerebellum of 3 normal Lewis rats (N), CFA-inoculated animals (CFA); EAE-diseased rats (EAE) or EAE-sensitised Lewis rats i.p. injected with MK-801 (0.3 mg/kg body weight) or vehicle (PBS, 1 ml/kg body weight) from day 7 to 12 P.I.(MK and V). CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with (A) anti-eNOS, (B) anti-bNOS and (C) anti-iNOS antibodies. NOS protein was detected using ECL (A) or PAP complex (B and C). Densitometric analysis of blots and SDS-gels (D) were undertaken and following correction for differences in total protein content NOS levels were expressed as % above normal value. Results shown are representative of 3 separate experiments.

7.3A). By comparison, vehicle treatment did not markedly alter eNOS expression in EAE-sensitised rats (41 % above normal) (Figure 7.3A).

bNOS expression was also increased in cerebellar cytosol preparations from EAE-inoculated Lewis rats (62 % above normal) (Figure 7.3B). Vehicle administration had minimal effect on cerebellar levels of bNOS in EAE-diseased animals (33 % above normal) but treatment with MK-801 enhanced enzyme expression in EAE-sensitised rats cytosol preparations (125 % above normal) (Figure 7.3B).

Levels of iNOS protein in the cerebellum of EAE-diseased animals were elevated (174 % above normal) (Figure 7.3C). Moreover, administration of MK-801 further increased iNOS expression in cerebellar cytosol preparations from rats sensitised for EAE (185 % above normal) (Figure 7.3C). In contrast no effect on EAE-inoculated rats cerebellar iNOS protein levels were observed following vehicle treatment (143 % above normal) (Figure 7.3C).

Although both bNOS and iNOS protein expression was enhanced in cerebellar tissue following MK-801 treatment of EAE-sensitised animals, nitrite levels in cerebellum cytosol samples were decreased (Figure 7.2). This may have resulted as a consequence of reduced nitrite production by the eNOS isoform as the cerebellar protein levels for this enzyme dropped below normal values following drug administration.

7.3.2 Effect of MK-801 Administration on NOS Protein Expression in the Medulla-Pons

eNOS, bNOS and iNOS protein expression was identified in medulla-pons cytosol preparations from all experimental groups examined (Figure 7.4). Furthermore MK-801 and vehicle administration had similar effects on NOS expression in medulla-pons tissue from diseased rats (Figure 7.3 and 7.4).

EAE-sensitised animals displayed elevated levels of all 3 NOS enzymes in cytosol samples from the medulla-pons (eNOS 180 %, bNOS 115 %, iNOS 54 % above normal) (Figure 7.4). Moreover, administration of vehicle had no marked effect on NOS expression in identical tissues (eNOS 156 %, bNOS 117 %, iNOS 57 % above normal) (Figure 7.4). However, treatment of EAE-inoculated rats with MK-801 resulted in

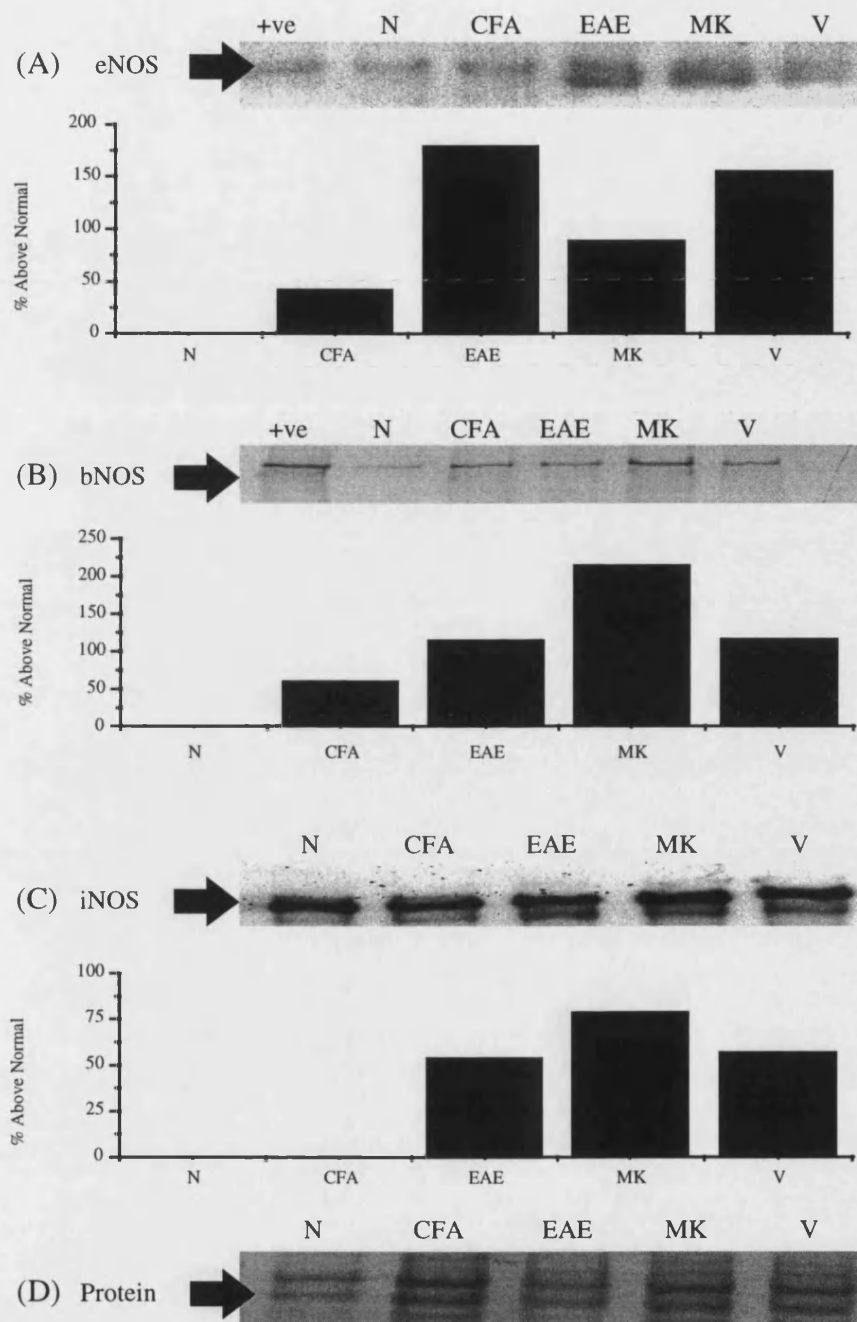


Figure 7.4 Effect of MK-801 administration on NOS protein expression

CNS cytosol was pooled from the medulla-pons of 3 normal Lewis rats (N), CFA-inoculated animals (CFA); EAE-diseased rats (EAE) or EAE-sensitised Lewis rats i.p. injected with MK-801 (0.3 mg/kg body weight) or vehicle (PBS, 1 ml/kg body weight) from day 7 to 12 P.I.(MK and V). CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with (A) anti-eNOS, (B) anti-bNOS and (C) anti-iNOS antibodies. NOS protein was detected using ECL (A and C) or PAP complex (B). Densitometric analysis of blots and SDS-gels (D) were undertaken and following correction for differences in total protein content NOS levels were expressed as % above normal value. Results shown are representative of 3 separate experiments.

decreased expression of eNOS (89 % above normal), but enhanced bNOS and iNOS protein levels (bNOS 215 %, iNOS 79 % above normal) in medulla-pons preparations (Figure 7.4).

Despite the fact that eNOS expression is reduced in the medulla-pons of MK-801 treated rats, nitrite levels in corresponding cytosol samples were not significantly altered. The decreased production of nitrite by eNOS may be overcome by enhanced nitrite generation from bNOS and iNOS as the expression of both enzymes was increased following administration of MK-801. Therefore, no overall effect on medulla-pons cytosol nitrite content in EAE-sensitised rats dosed with MK-801 would be observed.

7.3.3 Effect of MK-801 Administration on NOS Protein Expression in the Cervical Spinal Cord

All 3 NOS enzymes were identified in the cervical spinal tissue preparations examined (Figure 7.5). In addition, treatment of EAE-inoculated animals with MK-801 or vehicle had comparable effects on cervical spinal cord NOS levels as were detected in medulla-pons samples (Figure 7.3 and 7.4).

Increased levels of eNOS, bNOS and iNOS were present in cervical spinal cord cytosol fractions from EAE-diseased animals (eNOS 180 %, bNOS 194 %, iNOS 33 % above normal) (Figure 7.5). Furthermore, vehicle treatment did not dramatically alter NOS expression in cervical spinal cord tissue from EAE-sensitised rats (eNOS 190 %, bNOS 158 %, iNOS 31 % above normal) (Figure 7.5). By comparison, administration of MK-801 to EAE-inoculated animals increased the expression of b and iNOS (bNOS 484 %, iNOS 56 % above normal) but reduced levels of eNOS protein (49 % above normal) in cervical spinal cord preparations (Figure 7.5).

Although eNOS expression is decreased in cervical spinal cord tissue of EAE-diseased rats treated with MK-801 both bNOS and iNOS protein levels are enhanced. Therefore, differences in NO generation by each isoform may cancel each other out and hence explain why no significant reduction in nitrite levels incurred following drug administration.

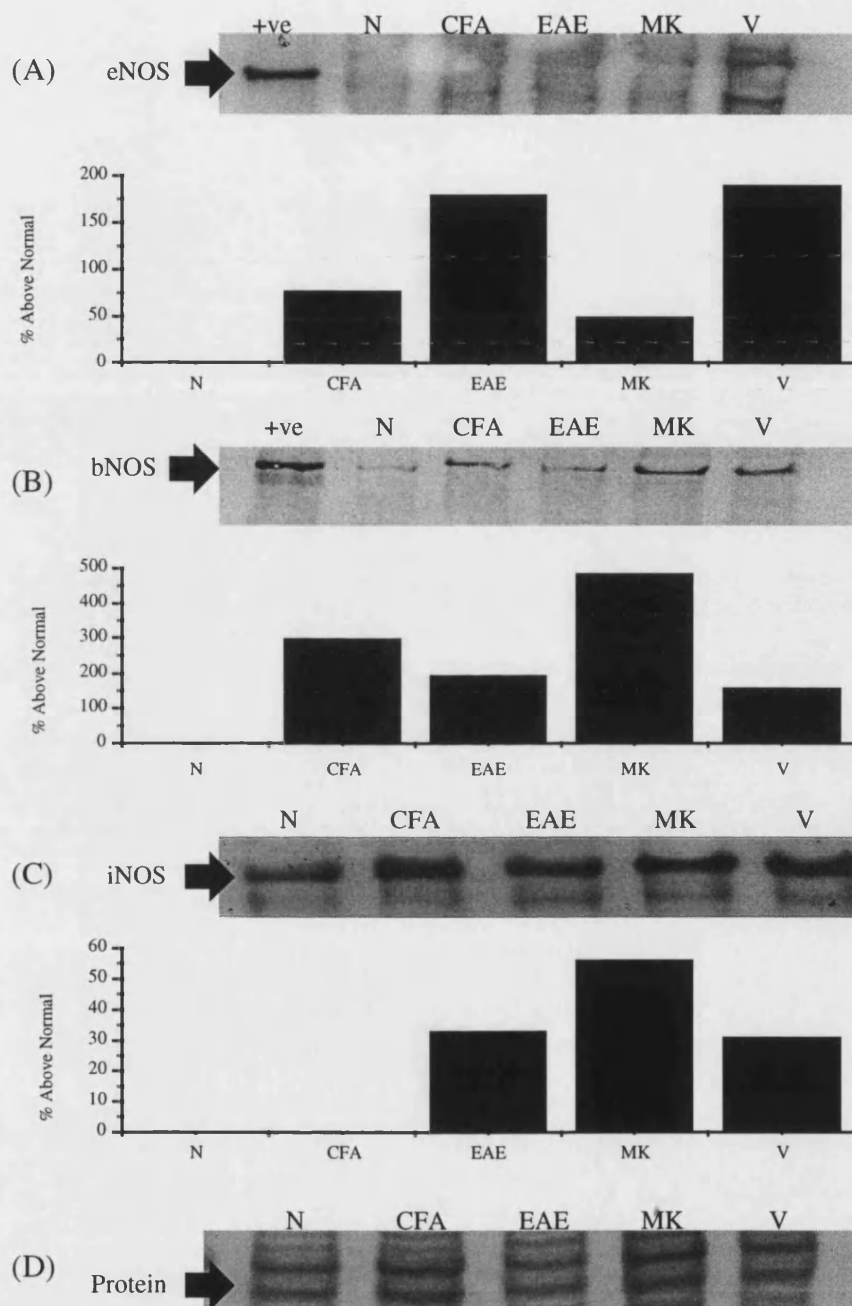


Figure 7.5 Effect of MK-801 administration on NOS protein expression

CNS cytosol was pooled from the cervical spinal cord of 3 normal Lewis rats (N), CFA-inoculated animals (CFA); EAE-diseased rats (EAE) or EAE-sensitized Lewis rats i.p. injected with MK-801 (0.3 mg/kg body weight) or vehicle (PBS, 1 ml/kg body weight) from day 7 to 12 P.I. (MK and V). CNS cytosol proteins were separated on SDS-gels and following electrophoretic transfer samples were blotted with (A) anti-eNOS, (B) anti-bNOS and (C) anti-iNOS antibodies. NOS protein was detected using ECL (A and C) or PAP complex (B). Densitometric analysis of blots and SDS-gels (D) were undertaken and following correction for differences in total protein content NOS levels were expressed as % above normal value. Results shown are representative of 3 separate experiments.

7.4 Effect of MK-801 Administration on ROS Levels in CNS Cytosol

In order to assess whether NMDA receptor antagonists prevent EAE by inhibiting the production of ROS, superoxide and hydrogen peroxide levels in CNS cytosol samples from MK-801 and vehicle treated rats were determined (Figure 7.6).

MK-801 administration significantly reduced EAE-diseased animals superoxide levels in the cerebella cytosol (4 ± 0.3 n moles superoxide/mg protein \pm S.E.M., $p < 0.01$, $n=5$) (Figure 7.6a). In addition, superoxide content of medulla-pons and cervical spinal cord tissue were also decreased upon treatment of EAE-inoculated rats with MK-801 (Figure 7.6a). However, vehicle administration reduced CNS cytosol superoxide levels in these preparations (Figure 7.6a).

Hydrogen peroxide levels were diminished in CNS cytosol fractions obtained from EAE-sensitised animals treated with MK-801 or PBS vehicle (Figure 8.6b). However, MK-801 administration significantly reduced hydrogen peroxide levels to below values recorded in vehicle treated animals in the cerebella tissue ($p < 0.01$) (Figure 8.6b).

7.5 Cellular Infiltration

The effects of MK-801 on cellular infiltration during EAE were assessed by determining the number of perivascular infiltrates in cervical spinal cord sections from drug and vehicle-treated animals (Figure 7.7). Administration of MK-801 significantly reduced cellular migration during EAE ($p < 0.01$) (Table 7.2). By comparison, vehicle treatment did not alter perivascular infiltration in spinal cord tissues (Table 7.2).

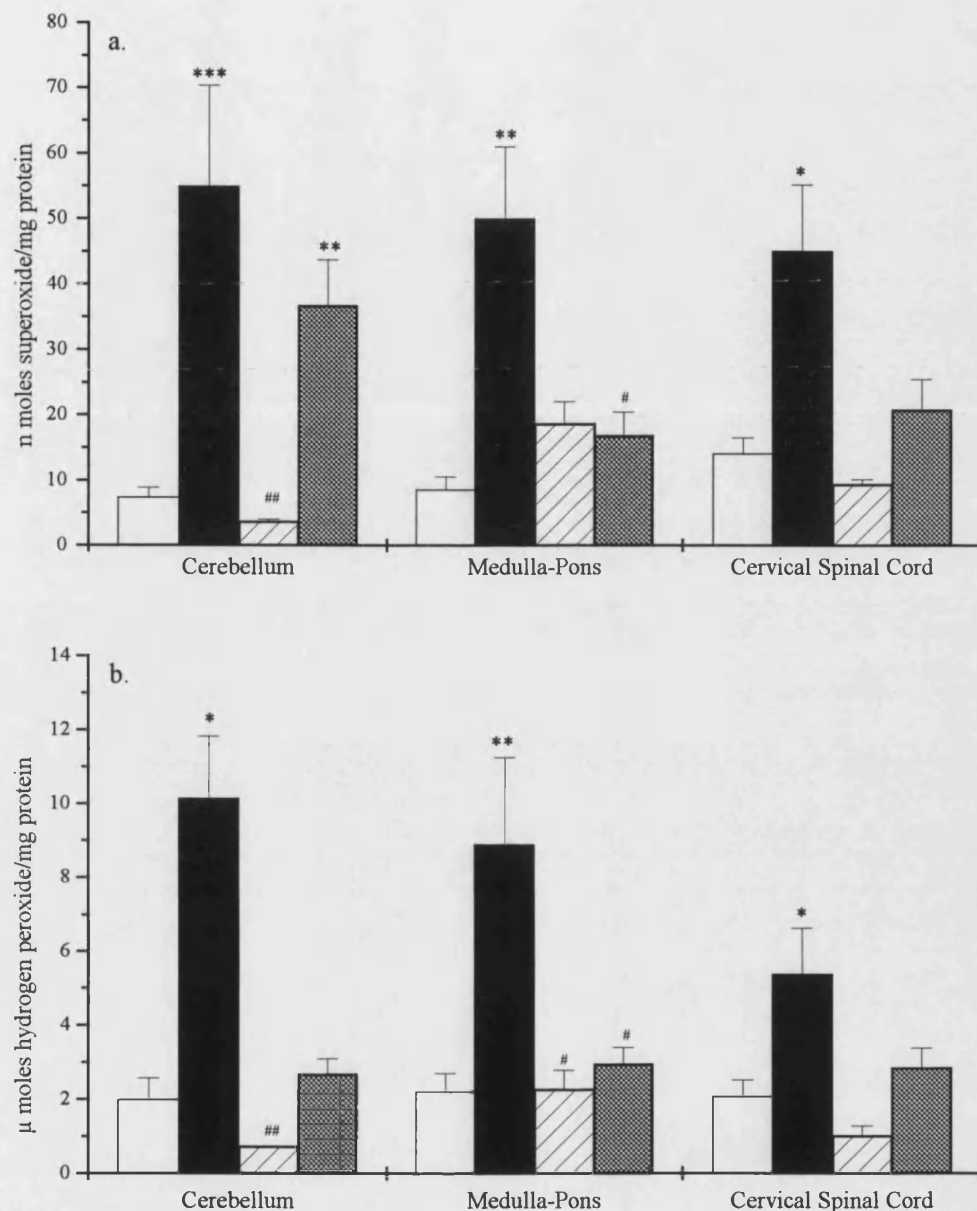


Figure 7.6 Effect of MK-801 administration on ROS levels in CNS cytosol
CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;
▨ EAE-sensitized Lewis rats i.p. injected with MK-801 (0.3mg/kg body weight) on days 7 to 12 P.I., n = 5; ▩ EAE-inoculated Lewis rats dosed i.p. with vehicle (PBS, 1ml/kg body weight) from day 7 to 12 P.I., n = 7.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Mann Whitney U test compared to normal animals. # $p < 0.05$, ## $p < 0.01$ Mann Whitney U test compared to EAE animals.

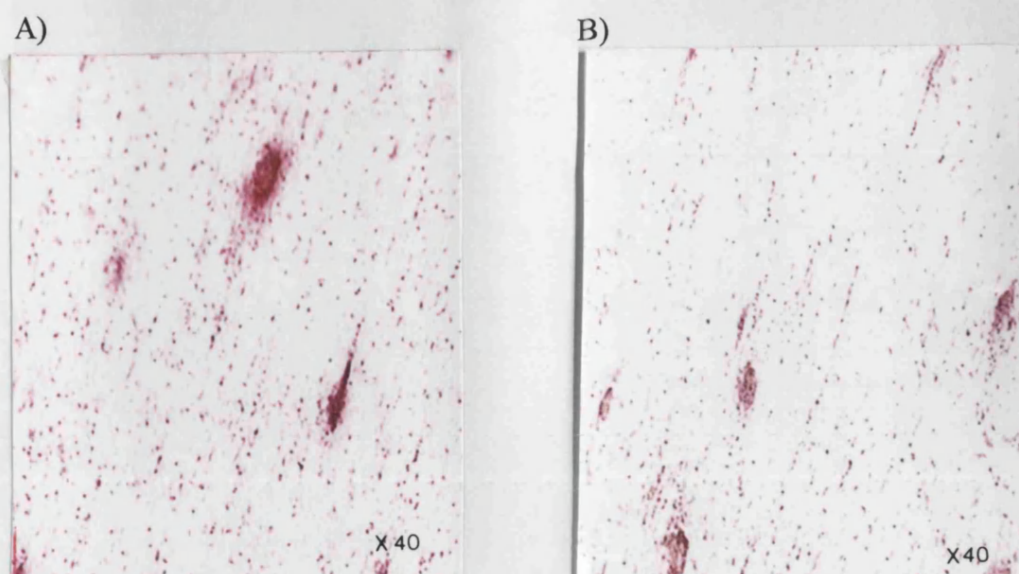


Figure 7.7 Effect of MK-801 on cellular infiltration during EAE

Cellular infiltration in haematoxylin and eosin stained cervical spinal cord samples from EAE-inoculated Lewis rats following treatment with A) MK-801 (0.3 mg/kg body weight, i.p. from day 7 to 12 P.I) or B) PBS vehicle (1 ml/kg body weight, i.p., from day 7 to 12 P.I.).

Table 7.2 Perivascular infiltrates in cervical spinal cord samples from EAE-sensitised Lewis rats following treatment with MK-801 (0.3 mg/kg body weight, i.p. from day 7 to 12 P.I) or vehicle.

Group	Mean Number of Lesions ^a /10 mm Section (± S.E.M.)
EAE	53 ± 4
MK-801	35 ± 2***
Vehicle	45 ± 3

^a Sections were cut at one standard depth, stained with haematoxylin and eosin and the total number of lesions per section was determined using light microscopy (x40). Values are means of 18 sections from 3 animals ± S.E.M. ***p<0.001 Mann Whitney U test compared to vehicle group.

7. DISCUSSION

7.1 NMDA Receptor Activation and EAE Pathogenesis

Neurodegeneration associated with overactivation of NMDA receptors is thought to be mediated by prolonged Ca^{2+} influx leading to a series of potentially neurotoxic events (Choi 1988). Influx of Ca^{2+} serves as a signal for the activation of the Ca^{2+} /calmodulin-dependent NOS (Bredt & Snyder 1992). In addition, elevated cytosolic free Ca^{2+} can activate phospholipase A_2 leading to the subsequent generation of ROS via arachidonic acid metabolism (Lafon-Cazal *et al.* 1993a). Both NO and ROS have been implicated in CNS damage (Dawson *et al.* 1993). Moreover, in the present study neurological EAE was characterised by an elevation in CNS nitrite and ROS levels. Therefore, NMDA receptor activation may be involved in the aetiology of EAE. Indeed, the presence of increased levels of NMDA receptor agonists have been reported in the CNS of animals during disease development (Honegger *et al.* 1989, Flanagan *et al.* 1995). Similarly the work of Wallström *et al.* (1996) demonstrating an inhibition of EAE following treatment with the glutamate receptor antagonist, memantine, provides further evidence of a role for NMDA receptor overstimulation in EAE. In the present investigation administration of the NMDA receptor antagonist, MK-801, abrogated the neurological development of EAE, thus reaffirming previous reports on the ability of NMDA receptor antagonists to alter the course of EAE (Wallström *et al.* 1996, Bolton & Paul in press), and suggesting an involvement of NMDA receptors in the neurological disease process.

7.2 Pathogenic Effects of NMDA Receptor Activation During EAE

Although other workers have proposed a role for NMDA receptor activation in EAE the precise pathological mechanisms involved have yet to be identified. Production of the cytotoxic free radical molecules, NO and ROS, following NMDA receptor activation may be implicated in disease pathogenesis. Interestingly nitrite and ROS levels were significantly reduced in the cerebellum of EAE-inoculated Lewis rats treated with

MK-801. However, no marked differences were observed in medulla-pons or cervical spinal cord nitrite and ROS content following administration of MK-801 or vehicle.

7.2.1 NO Formation

NO is produced in the CNS in a reaction catalysed by NOS. Activity of NOS is regulated by a number of factors including Ca^{2+} /calmodulin availability and the presence of cytokines. In addition polyamines have also been shown to affect NOS activity (Morgan 1994). Polyamines can competitively antagonise the activity of NOS as a result of their structural similarity to L-arginine. Activation of NMDA receptors not only increases NO generation but also induces the activity of ornithine decarboxylase, the enzyme responsible for polyamine synthesis (Porcell *et al.* 1991). Therefore the differential effects of MK-801 administration on CNS nitrite levels in EAE-diseased animals may result from a direct action on NOS activity or arise following an alteration in polyamine regulated NO production.

The expression of all 3 isoforms of NOS was determined in the CNS tissues of EAE-sensitised rats treated with MK-801. Furthermore similar profiles of NOS expression were identified in all CNS areas examined. Following drug administration eNOS protein levels decreased whereas nNOS and iNOS expression were enhanced in the CNS of EAE-diseased animals. As NMDA receptors are known to regulate the permeability of the BBB (Keonig *et al.* 1992), eNOS may be involved in this process. Therefore upon NMDA receptor inactivation by MK-801 levels of eNOS protein may be diminished with a resultant decrease in neurovascular permeability. Indeed this may explain, at least in part, the ability of MK-801 to prevent the neurovascular disruption associated with EAE (Bolton & Paul in press). Inhibition of NMDA receptor activation also gives rise to an upregulation of nNOS expression (Baader & Schilling 1996) thus accounting for the elevation in CNS levels of nNOS observed following drug treatment. Expression of iNOS protein is prevented by the presence of polyamines (Szabo *et al.* 1994). However, dosing EAE-inoculated rats with MK-801 has been shown to diminish the polyamine content of CNS tissues (Paul *et al.* 1997). Hence the inhibition of

polyamine synthesis may result in an increase in iNOS protein levels as demonstrated in this study. Although NOS expression was comparable in all CNS areas of EAE-sensitised animals following drug administration, nitrite levels were not. This implies that MK-801 affects the activity as well as the formation of NOS enzymes.

Cerebellar tissue contains higher levels of cNOS enzymes than any other area of the CNS (Förstermann *et al.* 1989). As cNOS isoforms are activated upon NMDA receptor stimulation antagonism with MK-801 may produce its greatest effect on NO generation in the cerebellum. In addition, drug administration did not alter the cerebellum levels of spermine and spermidine in EAE-diseased rats (Paul *et al.* 1997). These polyamines exert the greatest inhibitory effect on NOS activity (Hu *et al.* 1994). Therefore the presence in the cerebellum of inhibitory polyamines in conjunction with a prevention of NMDA-mediated Ca^{2+} influx would decrease NOS activity and result in a reduction in nitrite content. Another mechanism through which dosing with MK-801 could decrease nitrite levels in the cerebellum is via a reduction in the expression of NOS. Although eNOS protein levels were diminished in all CNS areas following MK-801 treatment cerebellum eNOS levels had fallen to below normal values. Hence cerebellum nitrite levels may be ultimately decreased.

By comparison the nitrite content of the medulla-pons and cervical spinal cord was not significantly altered upon MK-801 administration. This cannot be accounted for by differences in NMDA receptor density in various CNS areas as NMDA receptors have been identified in all areas of the CNS examined (Li *et al.* 1994, Wang *et al.* 1995). However variations in polyamine binding do exist between the CNS regions studied. Whereas polyamine binding sites are abundant in the medulla-pons much lower densities are observed in cerebellar tissue (Yoneda *et al.* 1991). The interaction of MK-801 and medulla-pons NMDA receptors may decrease polyamine formation which could prevent the inhibitory effects of polyamines on NO generation. Therefore nitrite production in the medulla-pons tissue of EAE-sensitised animals treated with MK-801 may remain elevated. Similarly, enhanced nitrite levels may persist in cervical spinal cord tissue due to a MK-801 mediated inhibition of the negative polyamine effects on NO formation. Moreover,

polyamine levels were reduced in both medulla-pons and cervical spinal cord from MK-801 treated EAE-diseased Lewis rats (C. Paul unpublished observations)

Clearly the study demonstrates that MK-801 does not inhibit the neurological development of EAE by preventing NO production. Hence an overproduction of NO subsequent to NMDA receptor activation is probably not involved in the pathogenic mechanisms of EAE. Indeed at present much controversy surrounds the exact role played by NO in NMDA-mediated neurodegeneration. Whereas several investigators have observed that inhibitors of NOS prevent glutamate-mediated cell death (Dawson *et al.* 1991, Izumi *et al.* 1992, Kolleger *et al.* 1993), other studies have not demonstrated such protective effects (Demerle-Pallardy *et al.* 1991, Pauwels & Leyson 1992, Hewett *et al.* 1993). Under certain circumstances NO mediates neurotoxicity associated with overstimulation of NMDA receptors. However, protective effects of NO have also been reported in the rat CNS (Kiedrowski *et al.* 1992, Lei *et al.* 1992). Recently both the neuroprotective and neurodestructive effects of NO have been identified in the same neuronal cultures (Kashii *et al.* 1996). The diverse effects of NOS inhibition on NMDA toxicity probably reflect the multifaceted roles of NO. Therefore, NO may have a dual role in NMDA receptor mediated toxicity. This may be accounted for by the fact that although NO participates in the intracellular cascade of events following NMDA receptor activation which lead to cell death (Dawson *et al.* 1993) it is also essential for providing an adequate blood supply in response to the increased metabolic demands (Faraci & Breese 1993). The final outcome will be dependent on the balance between these two processes (Globus *et al.* 1995). By comparison it has been proposed that the neuroprotective or neurodestructive ability of NO is dependent upon the redox state of the molecule with NO[•] and nitrosonium ion (NO⁺) mediating cytotoxic or cytoprotective effects respectively (Lipton *et al.* 1993).

7.2.2 ROS Production

In addition to NO formation, NMDA receptor stimulation generates ROS. Furthermore there is some uncertainty as to which of these free radicals plays the major role in NMDA mediated neuronal damage (Lafon-Cazal *et al.* 1993). As the protective effects of NMDA receptor antagonism in EAE were not mediated by a reduction in CNS levels of NO they may occur as a result of a decreased production of ROS. Indeed Lafon-Cazal and co-workers (1993b) demonstrated that oxygen radicals were the primary free radical involved in NMDA neurotoxicity. Moreover, MK-801 administration decreased the levels of both superoxide and hydrogen peroxide in the CNS of EAE-sensitised rats. However, similar effects were observed in the medulla-pons and cervical spinal cord following vehicle administration. Dosing EAE-inoculated animals with vehicle may alter endogenous corticosteroid levels which can in turn affect the generation of ROS

In conclusion the study demonstrates that NMDA receptor activation in EAE does not produce neurodegeneration via the production of free radicals. However other consequences of NMDA receptor activation may be implicated in the development of EAE. In particular NMDA induced cerebrovascular permeability alterations or polyamine formation may be important events in EAE aetiology.

7.2.3 BBB Breakdown

Although disruption in normal vascular permeability is a fundamental event in EAE little is known about the events which precipitate this loss of cerebrovascular integrity. NMDA receptors have been identified in the regulation of cerebroendothelial permeability associated with non-immune models of BBB dysfunction (Koenig *et al.* 1992, Dietrich *et al.* 1992, Miller *et al.* 1996b). Moreover, studies by other group members have demonstrated that administration of the NMDA receptor antagonist, MK-801, limits the BBB breakdown observed in EAE (Bolton & Paul in press). Neurovascular permeability may be decreased following antagonism of the NMDA receptor by preventing the formation of vasoactive agents. These include NMDA-mediated NO which is known to regulate cerebral blood flow and vascular tone (Faraci & Breese 1993) and has also been

implicated in glutamate-induced BBB disruption (Mayhan & Didion 1996). Furthermore, eNOS levels in the CNS of EAE-diseased animals were reduced upon MK-801 administration and this may facilitate the observed decrease in neurovascular permeability.

7.2.4 Polyamine Generation

Polyamine formation may also be implicated in the pathogenic mechanisms of NMDA receptor stimulation in EAE. EAE-sensitised animals were characterised by an elevation in CNS polyamine levels (Paul *et al.* 1997). However, these levels were reduced following MK-801 administration (Paul *et al.* 1997). This indicates an overproduction of polyamines in the CNS of EAE-diseased Lewis rats subsequent to NMDA receptor activation. Polyamines may contribute to the neurological development of EAE via two main mechanisms. In addition to direct cytotoxic effects in the CNS (Otsuki 1995), polyamines may also contribute to the BBB breakdown which initiates EAE (Koenig *et al.* 1983). Interestingly polyamine-induced neurotoxicity may partially involve NMDA receptor activation as pre-treatment with MK-801 has been shown to prevent CNS damage (Doyle & Shaw 1996). Additionally NMDA-receptor stimulation may generate polyamines, via ornithine decarboxylase activation, which exert cytotoxic effects on cerebroendothelial cells and induce neurovascular disruption (Koenig *et al.* 1989). Therefore it is likely that polyamines play an important role in the aetiology of EAE.

To conclude, the results of this investigation implicates a role for NMDA receptor activation in EAE pathogenesis. However the neurotoxic effects of NMDA receptor activation are not mediated by cytotoxic actions of NO or ROS. Neither do they involve an alteration in infiltrating inflammatory cells as cervical spinal cord perivascular infiltration in EAE-inoculated animals was unaffected by MK-801 treatment. Indeed the most likely pathogenic effects of NMDA receptor stimulation are disruption of the BBB and polyamine formation. Studies are ongoing to determine the precise mode of action through which NMDA receptors are involved in EAE.

CHAPTER 8

L-ARGININE

8. RESULTS

Inhibition of NO synthesis in the CNS of EAE-sensitised rats following administration of NOS inhibitors either had no effect on or aggravated neurological disease. Therefore, work was undertaken to determine the effect of increasing NO levels in the CNS on EAE. NO levels can be enhanced by administration of L-arginine substrate. Thus, L-arginine at a dose of 300 mg/kg body weight was administered orally in PBS vehicle to EAE-inoculated rats once daily for 12 days. In addition, normal animals were treated with L-arginine to ensure no adverse effects of increasing NO levels were induced. Following administration of L-arginine EAE-sensitised animals were assessed for effects on neurological signs and CNS levels of NO and ROS.

8.1 Effect Of L-Arginine Administration on Body Weight Loss and Neurological Development of EAE

L-arginine and vehicle-treated EAE-inoculated rats were examined daily for body weight changes and neurological disease signs (Figure 8.1 and Table 8.1). Untreated EAE-sensitised animals experienced a disease-related weight loss from day 10 P.I. onwards. This was followed by the onset of disease signs at day 12 P.I. By day 13 P.I. the majority of undosed EAE-diseased rats suffered paralytic symptoms (Table 8.1). Administration of L-arginine not only decreased the body weight loss experienced by EAE-inoculated animals at day 10 and 11 P.I. ($p<0.01$) but delayed the disease onset ($p<0.01$) (Figure 8.1. and Table 8.1). In addition, L-arginine treatment significantly abrogated disease severity ($p<0.01$) and reduced the number of animals showing paralysis ($p<0.05$) (Table 8.1). By comparison, vehicle administration had no effect on any of the disease parameters assessed. (Figure 8.1 and Table 8.1).

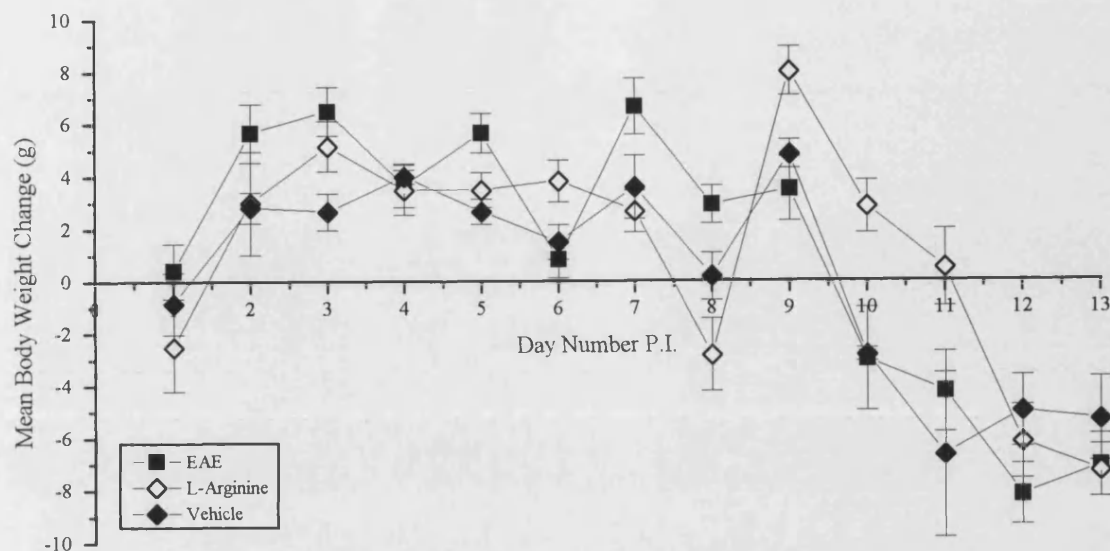


Figure 8.1 Effect of L-Arginine administration on body weight changes

Animals were assessed daily for changes in body weight which were measured in g and expressed as mean body weight change \pm S.E.M. (—■—) EAE-diseased Lewis rats, n=8; (—◇—) EAE-inoculated Lewis rats orally dosed with L-arginine (300 mg/kg body weight) from day 1 to 12 P.I., n=6; (—◆—) EAE-sensitised Lewis rats orally dosed with vehicle (PBS, 1 ml/kg body weight) from day 1 to 12 P.I., n=6.

Table 8.1 Neurological symptoms in EAE-sensitised Lewis rats after treatment with L-arginine (300 mg/kg body weight, orally, day 1 to 12 P.I.) or PBS vehicle (1 ml/kg body weight, orally, days 1 to 12 P.I.).

Group	Incidence Of EAE	Mean Day Of Disease Onset \pm S.E.M.	Mean Peak Disease Severity \pm S.E.M.	Number Of Rats Paralysed/Total
EAE	16/16	12 \pm 0.1	3 \pm 0.2	11/16
L-Arginine	6/6	13 \pm 0.3**†	1 \pm 0.3**†	1/6*
Vehicle	5/6	11 \pm 0.2	3 \pm 0.6	3/6

*p<0.05, **p<0.01 compared to EAE group.

†p<0.05 compared to vehicle group.

Differences in mean day of onset and mean peak disease severity were analysed using the Mann Whitney U test. Differences in incidence of disease and number of animals displaying signs of paralysis were analysed using the Chi-squared test.

8.2 Effect of L-Arginine Administration on Nitrite Levels in CNS Cytosol

CNS cytosol nitrite levels were measured to demonstrate whether L-arginine administration increased NO production. Cytosol prepared from the cerebellum, medulla-pons and cervical spinal cord of normal and EAE-sensitised animals treated with L-arginine and vehicle were assayed for their nitrite content (Figure 8.2).

Administration of L-arginine to normal rats resulted in elevated nitrite levels in all CNS areas examined with significant increases observed in the medulla-pons and cervical spinal tissues ($p < 0.05$) (Figure 8.2). In contrast treatment of normal rats with PBS vehicle did not significantly alter the nitrite content of CNS cytosol fractions (Figure 8.2).

Following L-arginine administration EAE-inoculated rats contained significantly enhanced amounts of nitrite in their CNS cytosol preparations (cerebellum $p < 0.01$, medulla-pons $p < 0.01$, cervical spinal cord $p < 0.01$) (Figure 8.2). However, vehicle treatment had no marked effect on CNS nitrite levels in EAE-diseased animals (Figure 8.2).

8.3 Effect of L-Arginine Administration on ROS Levels in CNS Cytosol

In the absence of L-arginine substrate the NOS enzymes can produce ROS instead of NO (Mayer *et al.* 1991). Therefore, the effect of L-arginine administration on ROS levels in the CNS of EAE-inoculated animals was examined (Figure 8.3).

Superoxide levels in cerebellar and medulla-pons cytosol samples from EAE-sensitised rats dosed with L-arginine were significantly reduced compared to values obtained in vehicle treated animals ($p < 0.01$) (Figure 8.3a). Although L-arginine administration reduced superoxide levels to within normal limits in cerebellar cytosol preparations, superoxide content in medulla-pons and cervical spinal cord samples remained above normal levels (Figure 8.3a). By comparison, no differences in superoxide content were observed in CNS cytosol of vehicle treated and undosed EAE-inoculated Lewis rats (Figure 8.3a).

Similar effects were observed on CNS cytosol hydrogen peroxide levels in EAE-inoculated animals following treatment with L-arginine (Figure 8.3b).

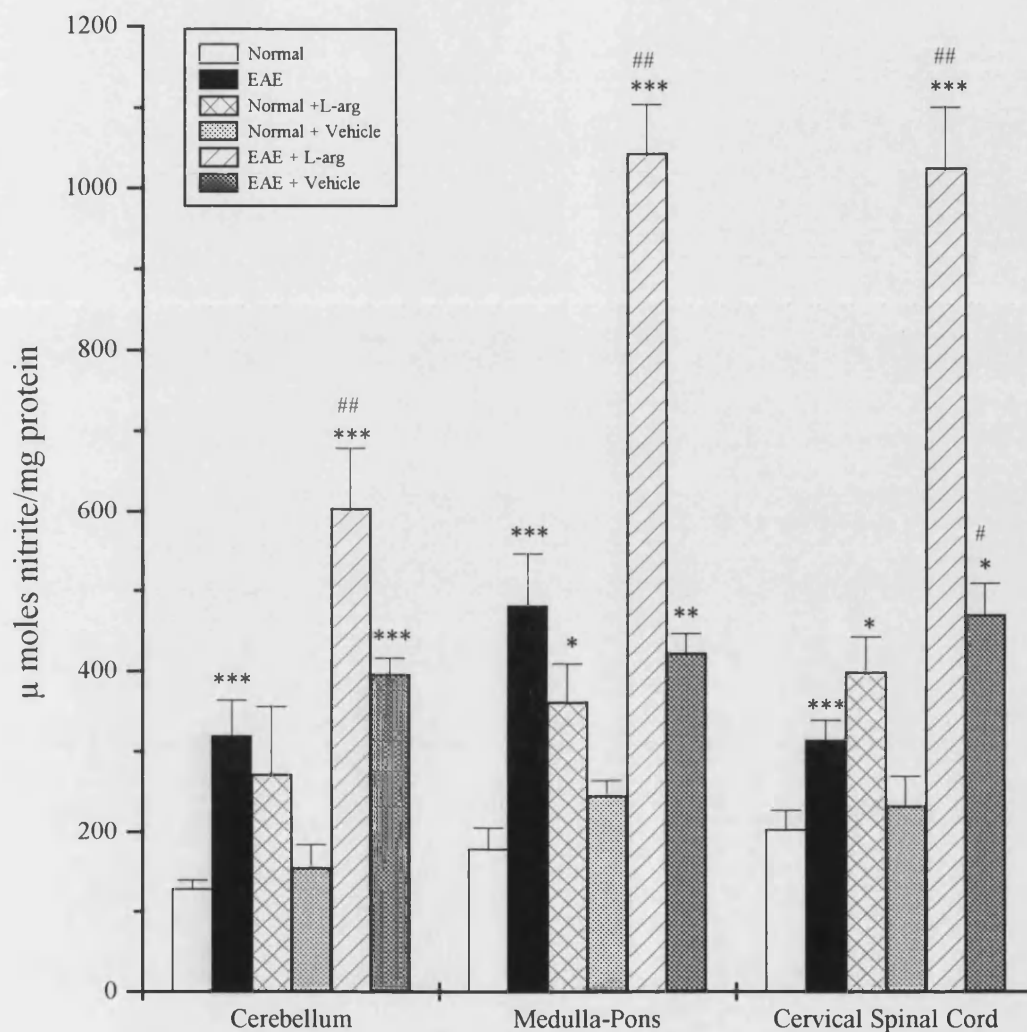


Figure 8.2 Effect of L-arginine administration on nitrite levels in CNS cytosol
 CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal tissues. Nitrite levels were determined using the Griess assay and expressed as μ moles nitrite/mg protein \pm S.E.M. following protein content estimation.
 Normal Lewis rats, n = 12; EAE-diseased Lewis rats, n = 12;
 Normal Lewis rats orally dosed with L-arginine (300 mg/kg body weight) for 12 days, n = 4; Normal Lewis rats orally dosed with vehicle (PBS, 1 ml/kg body weight) for 12 days, n = 4. EAE-sensitised Lewis rats orally dosed with L-arginine (300 mg/kg body weight) from day 1 to 12 P.I., n = 6; EAE-inoculated Lewis rats orally dosed with vehicle (PBS, 1 ml/kg body weight) from day 1 to 12 P.I., n = 6. * p < 0.05, ** p < 0.01, *** p < 0.001 Mann Whitney U test compared to normal animals. # p < 0.05, ## p < 0.01 Mann Whitney U test compared to EAE animals.

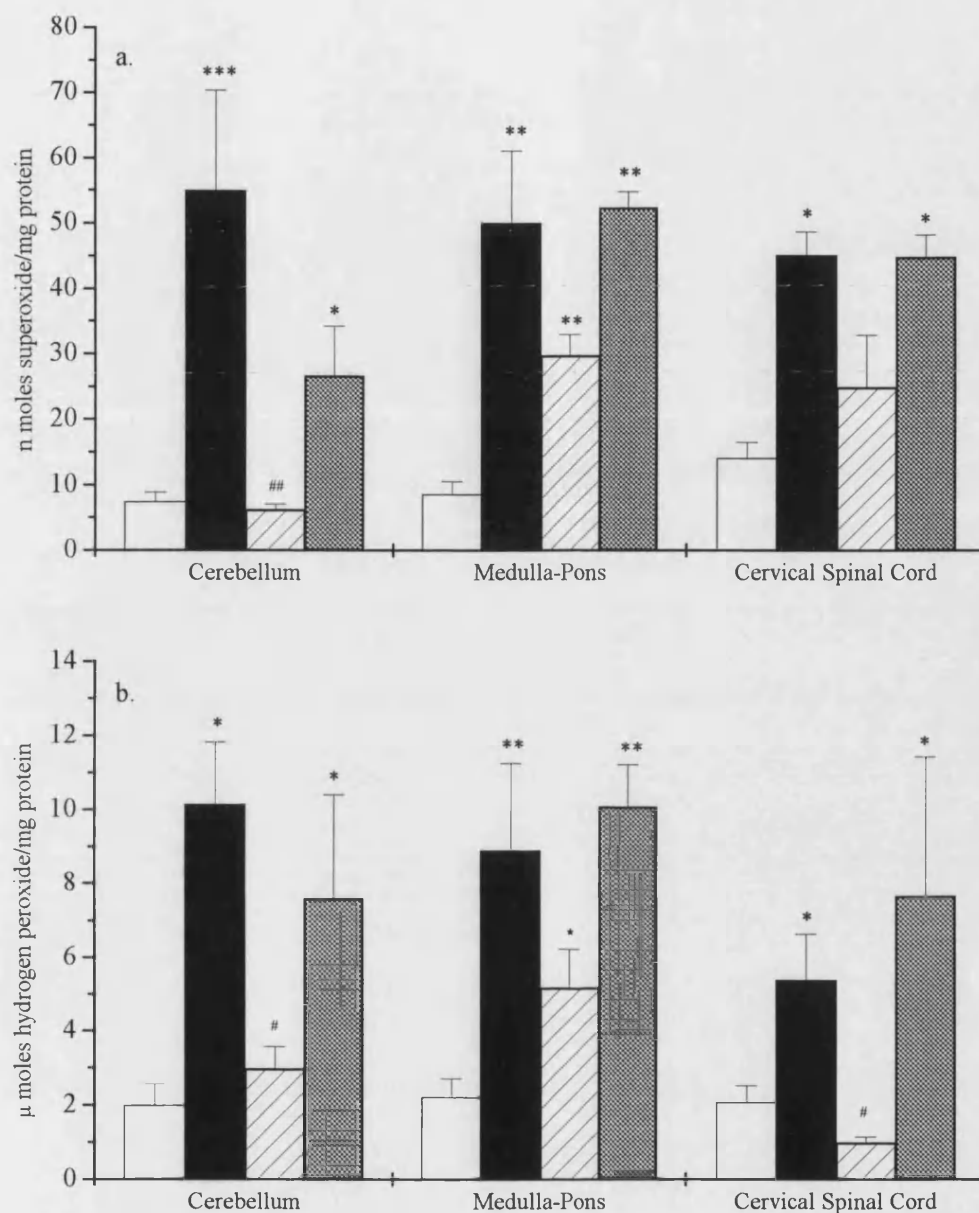


Figure 8.3 Effect of L-arginine administration on ROS levels in CNS cytosol
CNS cytosol was prepared from the cerebellum, medulla-pons and cervical spinal cord tissues.

a. Superoxide levels were determined using the reduction of ferricytochrome C and results were expressed as n moles superoxide/mg protein \pm S.E.M.

b. Hydrogen peroxide levels were measured by the oxidation of scopoletin and were expressed as μ moles hydrogen peroxide/mg protein \pm S.E.M.

□ Normal Lewis rats, n = 7; ■ EAE-diseased Lewis rats, n = 6;
▨ EAE-sensitized Lewis rats orally dosed with L-arginine (300mg/kg body weight) from day 1 to 12 P.I., n = 6; ▩ EAE-inoculated Lewis rats orally dosed with vehicle (PBS, 1ml/kg body weight) from day 1 to 12 P.I., n = 6.

*p<0.05, **p<0.01, ***p<0.001 Mann Whitney U test compared to normal animals. #p<0.05, ##p<0.01 Mann Whitney U test compared to EAE animals.

Administration of L-arginine to EAE-sensitised Lewis rats decreased hydrogen peroxide content in the CNS (Figure 8.3b). Moreover, hydrogen peroxide levels in L-arginine treated animals cerebellar and medulla-pons tissue were significantly reduced compared to values recorded in vehicle control rats ($p<0.05$). L-arginine administration reduced the amount of hydrogen peroxide in CNS cytosol from EAE-diseased rats cerebellar and cervical spinal tissues to within normal limits, however, medulla-pons hydrogen peroxide content continued to be greater than normal values (Figure 8.3b). In contrast, treatment of EAE-inoculated animals with PBS vehicle did not alter CNS cytosol hydrogen peroxide levels (Figure 8.3b).

8.4 Effect of L-arginine Administration on Cellular Infiltration

The effects of L-arginine on cellular infiltration during EAE were assessed by determining the number of perivascular infiltrates in cervical spinal cord sections from drug and vehicle treated animals (Figure 8.4). Administration of L-arginine or vehicle significantly reduced cellular infiltration during EAE ($p<0.05$) (Table 8.2). However, cell migration was significantly decreased in cervical spinal cord sections from animals dosed with L-arginine compared to samples from vehicle treated rats ($p<0.001$) (Table 8.2).

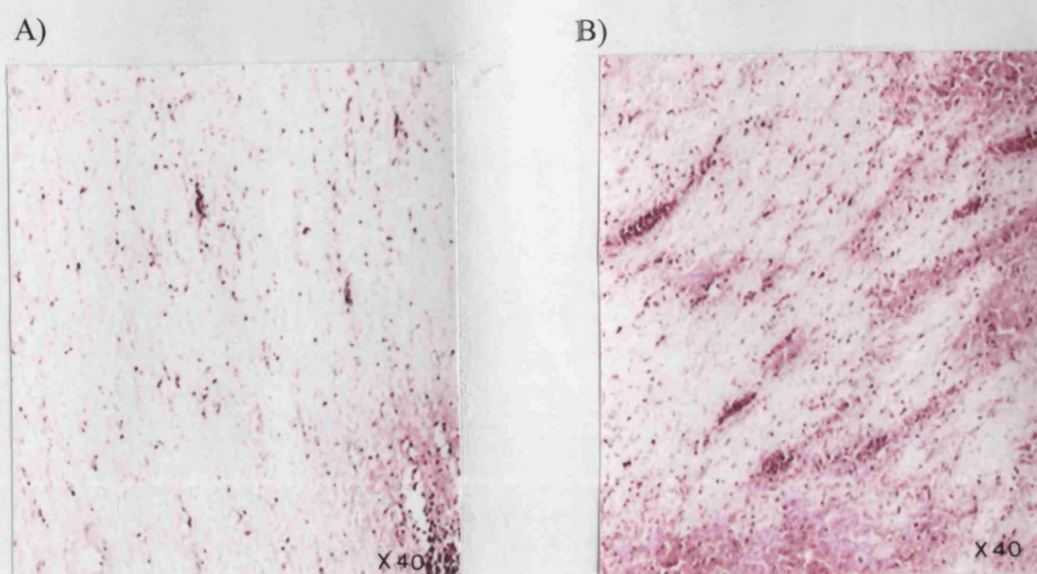


Figure 8.4 Effect of L-arginine administration on cellular infiltration during EAE
Cellular infiltration in haematoxylin and eosin stained cervical spinal cord samples from EAE-inoculated animals following treatment with A) L-arginine (300 mg/kg body weight orally from day 1 to 12 P.I) or B) PBS vehicle (1 ml/kg body weight, orally from day 1 to 12 P.I.)

Table 8.2 Perivascular infiltrates in cervical spinal cord samples from EAE-sensitised Lewis rats following treatment with L-arginine (300 mg/kg body weight, orally from day 1 to 12 P.I) or vehicle.

Group	Mean Number of Lesions ^a /10 mm Section (± S.E.M.)
EAE	53 ± 4
L-arginine	20 ± 1***†††
Vehicle	42 ± 3*

^a Sections were cut at one standard depth, stained with haematoxylin and eosin and the total number of lesions per section was determined using light microscopy (x40). Values are means of 18 sections from 3 animals ± S.E.M. *p<0.05, ***p<0.001 Mann Whitney U test compared to EAE group; ††† P<0.001 Mann Whitney U test compared to vehicle group.

8. DISCUSSION

8.1 Increasing NO Levels During EAE

As neurological EAE was unaffected or exacerbated following the reduction of NO by NOS inhibitor treatment, the effect of increasing CNS levels of NO on disease development was ascertained. NO levels can be enhanced by the administration of an NO-donor such as sodium nitroprusside or S-nitro-N-acetylpenicillamine (SNAP) (Baughton-Smith *et al.* 1990, Kubes & Granger 1992). However, due to the short half-life of these NO-donors this method of increasing NO was not appropriate for using in the EAE model. Therefore another method was devised to elevate CNS levels of NO. As L-arginine has been shown to reverse the competitive inhibition of NO (Whittle *et al.* 1995) and can be easily administered to animals via the oral route, rats were dosed with L-arginine to produce a model in which CNS levels of NO were increased. Indeed the ability of the drug to elevate NO was confirmed in the study by the observed enhancement in nitrite content of CNS tissues from normal animals treated with L-arginine.

In addition to effects on NO, L-arginine was administered to EAE-diseased rats to alter ROS production. Generation of ROS by NOS enzymes has been identified in the absence of adequate L-arginine substrate (Mayer *et al.* 1991, Heinzel *et al.* 1992). Since neurological EAE was associated with an elevation in the ROS content of the CNS, and increased levels of nNOS protein it was postulated that excess ROS may be produced by NOS as a consequence of an L-arginine deficiency during EAE. Therefore, examining the effects of L-arginine administration on EAE development and CNS levels of nitrite and ROS may allow better understanding of the precise role played by NO in the pathogenesis of EAE.

Interestingly, L-arginine treatment curtailed the onset and progression of neurological EAE. Furthermore nitrite levels were significantly elevated in the CNS of EAE-sensitised animals dosed with L-arginine. Therefore rather than being a cytotoxic molecule during EAE, NO may function in a neuroprotective capacity. Indeed other workers have also provided evidence for an immunosuppressive role for NO in EAE

pathogenesis (Ruuls *et al.* 1996). NO may prevent EAE progression through effects on key processes involved in the initiation of the disease. One of the characteristic features of EAE is the CNS infiltration of immunocompetent cells such as macrophages and lymphocytes (Brosnan *et al.* 1981). However, as NO functions as an anti-adhesion molecule excessive levels of NO may prevent the cellular infiltration observed in EAE (Kubes *et al.* 1991, Gaboury *et al.* 1993). Moreover, the number of perivascular infiltrates in spinal cord tissue from EAE-diseased rats was significantly reduced following L-arginine administration. Hence, the production of NO during EAE may limit the disease by altering the inflammatory cell composition of the CNS. In addition to effects on cell trafficking NO also prevents lymphocyte proliferation (Denham & Rauland 1992) and mast cell activation (Gaboury *et al.* 1996). As encephalitogenic T lymphocytes and mast cells play an important role in the aetiology of EAE (Bö *et al.* 1991, Levi-Schaffner *et al.* 1991, Bebo *et al.* 1996) inhibition of their proliferation and activation by NO respectively may prevent disease development. Clearly NO may suppress EAE through effects on the inflammatory cells implicated in disease pathogenesis.

8.2 Cytoprotective Role of NO Against Oxidant Injury

NO has also been observed to serve a protective role in pathological events associated with the excess production of ROS. Several studies have shown that stimulation of endogenous NO production or exogenous administration of NO inhibits oxidant-mediated tissue injury (Kubes *et al.* 1991, Choi 1993, Wink *et al.* 1993). Moreover, inhibition of NO synthesis enhanced injury in some models emphasising the protective role of NO in oxidant mediated cytotoxicity (Payne & Kubes 1993, Kavanagh *et al.* 1994). Pharmacological evidence exists implicating ROS in EAE pathogenesis (Bowern *et al.* 1984, Honegger *et al.* 1989, Lehmann *et al.* 1994, Ruuls *et al.* 1995). Furthermore the present investigation and other studies have demonstrated increased ROS production in tissues and cells isolated from the CNS of EAE-diseased animals (MacMicking *et al.* 1992, Ruuls *et al.* 1995, Scott *et al.* 1996). Therefore, the neuroprotective effects

observed in EAE as a consequence of L-arginine administration may result from an action of NO on ROS-dependent cytotoxicity.

In biological systems NO readily reacts with superoxide to yield highly toxic peroxynitrite (Beckman *et al.* 1990). However, interactions between NO and superoxide have also been observed to result in antioxidant activities (Kanner *et al.* 1991, Guitierrez *et al.* 1996). Indeed the outcome of this reaction appears to be critically dependent on the relative concentrations and rates of production of the two molecules (Darley-Usmar *et al.* 1995). Thus a ratio of 1:1 generates peroxynitrite and induces lipid peroxidation and cytotoxicity whereas an excess of NO can prevent this by scavenging peroxy radicals (Rubbo *et al.* 1994).

8.3 Neuroprotective Effects of L-Arginine Administration

The neuroprotective effects observed in EAE upon increasing endogenous NO formation by L-arginine treatment may occur through several mechanisms. Endogenous NO may exert a cytoprotective effect by reacting with haem, iron-sulphur and other metalloproteins to prevent the formation of powerful oxidant molecules which occurs as a result of the interaction between hydrogen peroxide and metals (Wink *et al.* 1994). In addition, NO may divert superoxide-mediated toxic reactions to other less damaging oxidative pathways (Gutierrez *et al.* 1996). Moreover, the diversion of superoxide through the peroxynitrite oxidation and decomposition pathways would also limit the accumulation and subsequent reactions of hydrogen peroxide by reducing the availability of superoxide for spontaneous or SOD-catalysed dismutation (Gutierrez *et al.* 1996). Interestingly, hydrogen peroxide levels were decreased in the CNS tissues of EAE-sensitised Lewis rats treated with L-arginine. Therefore, the endogenous NO formed in EAE-diseased animals upon the administration of L-arginine may reduce disease development by a suppression of hydrogen peroxide production. Additional effects of NO to prevent ROS-mediated cytotoxic reactions may occur via an inhibition of oxidant-induced lipid peroxidation (Rubbo *et al.* 1994). Finally NO may transcriptionally activate

expression of antioxidant enzymes and so reduce the toxic potential of ROS (Gutierrez *et al.* 1996).

In addition to its ability to enhance NO production and scavenge ROS to prevent pathological effector mechanisms, L-arginine administration may also reduce ROS toxicity through NO-independent effects. It has been previously shown that in the absence of L-arginine ROS are generated by NOS (Heinzel *et al.* 1992, Xia *et al.* 1996). Therefore, the enhanced CNS production of ROS observed in EAE may occur as a consequence of L-arginine depletion. The availability of adequate L-arginine substrate for the NOS catalysed formation of NO may be diminished in EAE due to the recently reported increased synthesis of polyamines (Paul *et al.* 1997). Although the precursor for polyamine biosynthesis is ornithine this may be derived from L-arginine (Morgan 1994). Hence during EAE the enhanced synthesis of polyamines may lead to an indirect reduction in L-arginine. Administration of exogenous L-arginine would therefore counteract this depletion and consequently decrease ROS generation by NOS. Indeed the CNS production of both superoxide and hydrogen peroxide were reduced in EAE-diseased rats following treatment with L-arginine. Although this decrease in CNS levels of ROS may occur as a consequence of enhanced NO formation, as discussed above, it may also result from the increase in endogenous L-arginine availability preventing ROS formation upon NOS activation.

Clearly the results of this study demonstrate that enhanced endogenous NO and decreased ROS levels contribute to limiting the neurological development of EAE. Hence this work suggests that NO production in EAE exerts a cytoprotective rather than a cytotoxic effect which complicates pharmacological investigations into the role of NO in EAE. Indeed further work must be undertaken to identify the precise role played by NO and ROS in EAE pathogenesis.

CHAPTER 9

CONCLUSIONS

9.1 NO and EAE

The present investigation was undertaken to clarify the role of NO in EAE pathogenesis. Initially the importance of NO in disease aetiology was determined by examining nitrite levels and NOS protein expression in the CNS at various stages of EAE. Nitrite and NOS protein levels were elevated in CNS tissues from EAE-sensitised rats throughout the induction and effector phases of the disease indicating a possible involvement of NO in EAE pathogenesis. However, CNS nitrite content and protein expression were also increased during the recovery stage of EAE which suggests that NO may be important in disease regulation. Therefore, the results of the study indicate that NO may have a dual role in EAE.

NO may exert cytotoxic effects in the pathogenesis of EAE via several mechanisms. Toxicity of NO in the CNS may be due to inhibition of cellular mitochondrial respiration (Cleeter *et al.* 1994, Zielasek *et al.* 1995) or DNA synthesis and repair (Cosi *et al.* 1994). Alternatively, NO formation may contribute to neurodegeneration by activation of cyclooxygenase (Corbett *et al.* 1993) or stimulation of TNF- α production (Magrinat *et al.* 1992). Furthermore, NO may react with superoxide to form the highly toxic molecule, peroxynitrite (Beckman *et al.* 1990) which can cause CNS damage via lipid peroxidation (Radi *et al.* 1991) or loss of cellular integrity (Lipton *et al.* 1993).

Neuroprotective effects of NO during EAE may be mediated through various mechanisms. NO may suppress EAE through effects on the inflammatory cells implicated in disease pathogenesis. More specifically NO may prevent T lymphocyte proliferation (Denham & Rauland 1992) and mast cell activation (Gaboury *et al.* 1996), both of which may be important in EAE (Bö *et al.* 1991, Levi-Schaffner 1991, Bebo *et al.* 1996). Recently Okuda *et al.* (1997) have proposed that NO may exert immunosuppressive effects in EAE by the elimination of CNS inflammatory cells through apoptosis. Additionally NO may serve a protective role in EAE by reacting with ROS. Although the reaction between NO and superoxide yields peroxynitrite (Beckman *et al.* 1990) interactions between these two molecules have also been observed to result in antioxidant

activities (Kanner *et al.* 1991). Indeed the outcome of the reaction appears to be dependent on the relative concentrations and rates of production of NO and superoxide (Darley-Usmar *et al.* 1995) with a ratio of 1:1 generating cytotoxic peroxynitrite and an excess of NO preventing toxicity by scavenging peroxy radicals (Rubbo *et al.* 1994).

9.2 Pharmacological Manipulation of NO in EAE

In order to further define the precise role of NO in the aetiology of EAE the effects of pharmacological manipulation of CNS NO levels on disease development were assessed. Interestingly administration of immunosuppressive agents such as Dex or CsA inhibited nitrite production in the CNS and abrogated EAE strongly indicating an involvement of NO in disease pathogenesis. However, treatment of EAE-inoculated rats with specific NOS inhibitors, while decreasing CNS nitrite levels, had variable effects on neurological disease signs. Administration of the bNOS inhibitor, 7-NI, prevented EAE, the iNOS inhibitor, AG, had no effect on disease development. Moreover, appropriate vehicle treatment elicited similar responses. By comparison, dosing sensitised animals with the non-selective NOS inhibitor, L-NAME, aggravated EAE suggesting a suppressive role for NO in the disease. The involvement of NO in EAE pathogenesis was further complicated following the administration of the NMDA receptor antagonist, MK-801, to EAE-inoculated rats. Although MK-801 inhibited EAE the drug did not decrease nitrite formation in all areas of the CNS indicating that the suppressive effects of MK-801 treatment on disease development may not be mediated via NO inhibition. Finally, EAE-sensitised animals were dosed with L-arginine to determine the effect of increasing CNS levels of NO on neurological disease progression. L-arginine administration both enhanced CNS nitrite content and abrogated EAE thus providing additional evidence for a suppressive role of NO in disease pathogenesis.

9.3 Interactions Between ROS and NO During EAE

The proposed dual effects of NO as a cytotoxic or cytoprotective molecule during EAE may be due to interactions of the molecule with ROS. Therefore, the ROS content of CNS tissues from EAE-diseased animals was determined in the presence and absence of drug administration to elucidate the effect of altering NO levels on ROS production. Although both superoxide and hydrogen peroxide levels were elevated in the CNS of sensitised animals only the superoxide increase appeared to be a disease specific effect. Interestingly, superoxide content of the CNS was only enhanced prior to the onset of EAE suggesting a possible role for superoxide as a pathogenic effector molecule in the disease process. Furthermore, as CNS tissue nitrite content was also increased prior to neurological disease development it may be that the NO and superoxide react to form cytotoxic peroxynitrite thus accounting for some of the cytotoxic properties of NO in EAE. Indeed recent work by Hooper *et al.* (1997) has implicated peroxynitrite in EAE pathogenesis. In contrast the interaction of NO and superoxide during EAE may serve a neuroprotective function. Although superoxide levels were reduced in the CNS during the active and recovery phases of EAE, nitrite content of the tissue was enhanced. Therefore the excess NO in the CNS may scavenge superoxide and so prevent cytotoxic reactions.

During EAE the potential exists for the interaction of NO and ROS to result in cytotoxic or cytoprotective effects. To ascertain whether NO scavenges superoxide during EAE the consequences of inhibiting CNS production of NO on ROS levels were assessed. The results could not be clearly interpreted due to the ability of the pharmacological agents employed to prevent ROS formation in addition to inhibiting NO synthesis. However the inhibitory effects of L-arginine administration on EAE development were associated with an increase in CNS nitrite levels but a reduction in ROS content of the tissue implicating that NO may scavenge superoxide during the disease.

9.4 Future Directions

Although the present investigation provides evidence demonstrating a potential role for NO as an effector molecule in both the development and resolution of EAE further work is required to elucidate the importance of these actions in the overall disease aetiology. The relative contribution of NO as a cytotoxic or cytoprotective molecule in the CNS during EAE may be defined completing the following objectives:

- devise a reliable method for measuring peroxynitrite production in CNS tissues to determine differences between samples from normal and EAE-diseased animals.
- administer peroxynitrite inhibitors/scavenger such as uric acid, nicotinic acid and desferoxamine to EAE-sensitised rats and assess the effect on disease development.
- measure L-arginine concentrations, by HPLC, in the CNS during the disease progression to ascertain if a deficiency in the NOS substrate contributes to the generation of cytotoxic ROS in EAE.
- utilise more selective NOS inhibitors, such as S-methyl-thiocitrulline, N^G-nitro-L-arginine and L-N⁶-(1-iminoethyl)lysine and measure NOS activity in the CNS to define which isoform/s of NOS are important in the disease.
- examine the possible mechanisms through which NO may exert immunosuppressive effects during EAE by assessing the lymphocyte proliferation and mast cell activation following administration of NOS inhibitor.

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APPENDIX

MATERIALS

Material	Source
β -mercapthoethanol	BDH, Poole, UK
7-Nitroindazole	Lancaster, Lancashire, UK
Acetic acid	Fisons, Loughborough, UK
Acetone	Fisons, Loughborough, UK
Acrylamide	BDH, Poole, UK
Ammonium nickel sulphate	Sigma, Poole, UK
Ammonium persulphate	BDH, Poole, UK
anti-bNOS antibody	Affiniti Research Products Ltd, Exeter, UK
anti-eNOS Antibody	Affiniti Research Products Ltd, Exeter, UK
anti-iNOS antibody	Affiniti Research Products Ltd, Exeter, UK
BICINE	Sigma, Poole, UK
Bovine serum albumin	Sigma, Poole, UK
Bromophenol blue	Fisons, Loughborough, UK
Calcium chloride	Fisons, Loughborough, UK
Catalase	Sigma, Poole, UK
Cellulose phosphate	Sigma, Poole, UK
Cobalt chloride	Sigma, Poole, UK
Coomassie brilliant blue	Sigma, Poole, UK
Copper sulphate	Sigma, Poole, UK
Cyclosporin A	gift from Sandoz, Switzerland
Dexamethasone	Organon Laboratories, Cambridge, UK
Diaminobenzidine	Sigma, Poole, UK
Dithiothreitol	Sigma, Poole, UK
ECL reagents	Amersham, Buckinghamshire, UK
EDTA	Sigma, Poole, UK

Eosin	Sigma Diagnostics, St. Louis, USA
Ethanol	Fisons, Loughborough, UK
Extra virgin olive oil	J Sainsubrys, UK
Ferricytochrome C	Sigma, Poole, UK
Folin's Ciocalteu reagent	BDH, Poole, UK
Freund's incomplete adjuvant	Difco, Detroit, USA
Glycine	Sigma, Poole, UK
Goat anti-mouse IgG	DAKO, Denamrk
Haematoxylin	Sigma Diagnostics, St.Louis, USA
Halothane	Rhône Mérieux Ltd., Harlow, UK
Horseradish peroxidase	Sigma, Poole, UK
Horseradish peroxidase linked sheep anti-mouse Ig	Amersham, Buckinghamshire, UK
Human aortic endothelial cell lysate	Affiniti Research Products Ltd, Exeter, UK
Hydrogen peroxide	Sigma, Poole, UK
L-arginine	Sigma, Poole, UK
L-NAME	Sigma, Poole, UK
Magnesium chloride	Fisons, Loughborough,UK
Methanol	Fisher Scientific Int., Loughborough, UK
Milk powder	Marvel, Premier Beverages, Stafford,UK
MK-801	gift from Merck Sharp & Dohme, UK
Mouse macrophage cell lysate	Affiniti Research Products Ltd, Exeter, UK
Mouse monoclonal PAP	Sigma, Poole, UK
<i>Mycobacterium tuberculosis</i>	Difco, Detroit, USA
N-naphthylethylenediamine dihydrochloride	Sigma, Poole, UK
NN'-methylenebisacrylamide	BDH Ltd., Poole,UK
Peanut oil	Sigma, Poole, UK

Phosphate buffered saline	Sigma, Poole, UK
Phosphoric acid	Sigma, Poole, UK
PVDF membrane	Millipore, Watford, UK
Rat pituitary tumor cell lysate	Affiniti Research Products Ltd, Exeter, UK
Scopoletin	Sigma, Poole, UK
Sodium azide	Sigma, Poole, UK
Sodium carbonate	BDH Ltd., Poole, UK
Sodium hydroxide	Fisons, Loughborough, UK
Sodium dodecyl sulphate	Sigma, Poole, UK
Sodium nitrite	Sigma, Poole, UK
Sodium sulphate	Sigma, Poole, UK
Sodium tartrate	Sigma, Poole, UK
Sucrose	Fisher Scientific Int., Loughborough, UK
Sulphanilamide	Sigma, Poole, UK
Superoxide dismutase	Sigma, Poole, UK
TEMED	Sigma, Poole, UK
Tris	Sigma, Poole, UK
Triton X-100	Sigma, Poole, UK
Tween 20	Sigma, Poole, UK
Xylene	BDH Ltd., Poole, UK